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Chemopreventive Agent

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13. ABSTRACT (Maximum 200 Words)

We identified a novel vitamin D analog, 1α-hydroxy-24 ethyl vitamin D5 (1α(OH)D5) that showed potent growth inhibitory and cell-differentiating actions in breast cancer cells. Based on our findings in vitro and in vivo, we hypothesized that 1α(OH)D5 (D5), when administered to women with breast cancer, will induce differentiation of dedifferentiated cells and thereby prevent progression of malignancy. In 1999-2000, we completed preclinical studies in rats, showing that D5 has no serious toxicity; high doses led to a hypercalcemic effect, which was reversible. In vitro studies showed that D5 has no effect on normal breast epithelial cells but induces apoptosis in breast cancer and showed apoptotic effect in fibroadenomas. In 2000-2001, under GMP, we completed preclinical toxicity studies in dogs and completed synthesis of 1α(OH)D5. In vitro studies suggested that D5 has no effect on normal breast tissues. In 2001-2002, in vitro studies showed D5 to have no effect of cell proliferation , cell death, or differentiation markers (casein) in nonmalignant breast epithelial cells. In 2002-2003, in vitro studies suggested a differential effect of D5 on ER+ vs. ER- cells and that D5's action may be mediated, in part, by VDR. Clinical trial protocols were updated for both the UIC IRB and FDA. In 2003-2004, the clinical protocol was updated and approved by the UIC IRB, and Lutheran General Hospital was removed from the protocol. Currently, all of the preclinical toxicology and pharmacology studies have been completed and an IND application has been submitted to the FDA. The FDA has asked for some additional

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Table of Contents

Introduction

Vitamin D and its analogs have shown potential chemopreventive and chemotherapeutic effects on various malignant tumors (1-14). The active metabolite of vitamin D3, 1,25(OH)2D3, has been shown conclusively to induce differentiation in vitro in a variety of cancer cells, including breast cancer cells (12-14). 1,25(OH)2D3 is hypercalcemic, and thus its use as a preventive and therapeutic agent is limited. Although a number of vitamin D analogs are synthesized, only limited vitamin D-related compounds have reached clinical trial. Recently, we identified a vitamin D analog that showed potent growth inhibitory and cell-differentiating action in breast cancer cells. The effects of 1α(OH)D5 were extensively investigated in vitro and in vivo. We aim to pilot 1α(OH)D5 from an experimental laboratory model to the clinical setting. The effects of 1α(OH)D5 were investigated extensively in in vitro and in vivo experimental models, and some pronounced effects of 1α(OH)D5 are summarized below.

♦ 1α(OH)D5 has chemopreventive action in mouse organ culture model (15).

♦ 1α(OH)D5 has chemopreventive action on DMBA-induced mammary tumors in rats (16).

♦ 1α(OH)D5 has both growth inhibitory and cell-differentiating actions in human breast carcinoma cells (17,18).

♦ 1α(OH)D5 supplemented in the diet inhibits the in vivo growth of human breast carcinoma transplanted in athymic mice (18).

♦ 1α(OH)D5 is metabolized into two major metabolites (1,24 and 1,25 vitamin D5) in human breast tumors and nonmalignant breast tissues.

♦ Preclinical toxicity studies have been completed in two different species. Studies were performed in male and female rats and dogs under GLP. Adult male and female rats/beagle dogs were given 1-10 μg/kg body weight 1α(OH)D5 by gavage for 28 consecutive days. 1α(OH)D5 in rats showed no serious toxic effect. No animals died during the course of study, and no adverse treatment-related clinical signs of toxicity were observed. Increased serum calcium levels were observed in both sexes at the high dose level and in females at mid-dose levels. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at mid- and high-dose levels, and in females at all doses (19).

♦ The effect of 1α(OH)D5 was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group (19).

♦ Studies were done on the in vitro effect of 1α(OH)D5 on malignant and nonmalignant tissues obtained from breast cancer patients at the time of surgery. 1α(OH)D5 had no effect on cell proliferation, cell death, or differentiation markers (casein) in nonmalignant breast tissues (epithelial cells). 1α(OH)D5 induced cell death in fibroadenomas. In malignant tumors, 1α(OH)D5 induced apoptosis (20). Preclinical toxicity studies in dogs and rats suggested that the compound is well tolerated and causes no serious toxicity.

♦ In the current funding period, Lutheran General Hospital was removed from the study protocol. We have designed a clinical protocol for phase I clinical studies in breast cancer patients. The clinical protocol has been approved by the UIC IRB and the informed consent

form is approved. We have submitted an application to the FDA for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA has requested a stability study of 1α(OH)D5 under GMP/GLP conditions. We have given a contract to an FDA-approved laboratory to provide us with this data. It appears that once the stability data is received, the FDA will approve our application. Following FDA approval, we will provide all the necessary papers to the DOD committee on human experimentation. It is expected that a "go ahead" will be received from DOD and the clinical trial will be initiated.

Hypothesis proposed

We hypothesize that (1) 1α(OH)D5 administered to women with breast cancer will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy, and (2) in women with premalignant lesions, 1α(OH)D5 will prevent dedifferentiation and thus prevent induction and/or development of breast cancer.

Technical Objectives proposed

The specific objectives of the proposed study are to:

1. Establish and evaluate biomarkers predicting 1α(OH)D5 response in malignant breast cancer and DCIS (Ductal Carcinoma in Situ).

2. Study the molecular mechanism by which 1α(OH)D5 induces differentiation/inhibits proliferation of breast cancer cells.

3. Perform (according to FDA requirement) preclinical toxicity and pharmacokinetic studies of 1α(OH)D5.

4. Initiate a phase I/II trial in advanced breast cancer patients. (During this trial, we will also obtain data on the metabolism of 1α(OH)D5 in humans.)

Successful completion of the proposed study will identify a new chemotherapeutic and possibly chemopreventive agent in breast cancer.

Results

Effect of 1α(OH)D5 on human breast carcinoma.

We examined the effect of 1α(OH)D5 in human breast carcinoma tissues incubated in vitro in control and 1 μM 1α(OH)D5-containing medium. In most of the malignant breast tumors studied, the original histopathological features were preserved up to 48 hours when tissues were incubated in the control medium. Very few cells in this control tissue showed apoptotic or pyknotic changes. In contrast, cells incubated for 48 hours in medium containing 1 μM 1α(OH)D5 contained apoptotic cells. In addition, many cells at various stages of apoptotic death were observed. During the current funding period, the cell proliferative index was determined by staining tissue sections with Ki67. Results indicated that there was no effect of 1α(OH)D5 on the cell proliferation of the normal breast epithelial cells in culture whereas the Ki67 staining was considerably reduced in the cancer tissues.

In previous studies, we showed that, among the various breast cell differentiation-associated biomarkers studied, increased α2 integrin and casein levels were found to be the most reliable and sensitive parameters indicating response to 1α(OH)D5. We studied alpha2 integrin

expression in paraffin sections of human breast carcinomas and nonmalignant breast tissues. Although the alpha2 antibody used in our studies is highly recommended for immunohistochemistry, we were unable to observe specific staining for integrin in any tissues studied. We tested several antigen retrieval systems (citrate buffer, protease digestion, trypsin digestion, SDS treatment, microwave techniques) with non-reliable results. Currently, we are analyzing alpha2 integrin expression in frozen tumor/nonmalignant breast tissues.

Nonclinical Pharmacology

The effects of 1α(OH)D5 were evaluated in a variety of experiments. Most of these results have been published (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73; Mehta RR et al. Breast Cancer Res Treat. 1993;25(1):65-71; Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6) Four models were employed to evaluate the efficacy of these agents. These include: 1) estrogen receptor (ER)-positive and ER-negative breast cancer cell lines; 2) mouse mammary gland organ culture (MMOC); 3) chemically induced rat mammary cancer; and 4) xenograft transplant models bearing human breast cancers. The following sections describe the results generated from these experiments.

Antiproliferative activity against well established breast cancer cell lines in vitro.

The effects of 1α(OH)D5 were evaluated on the proliferation of several breast cancer cell lines with known estrogen receptor (ER), progesterone receptor (PR), and vitamin D receptor (VDR) status. As shown in Table 2, these included MCF7 (ER+, PR+, VDR+), T47D (ER+, PR+, VDR+), ZR75A (ER+, PR+, VDR+), BT474 (ER+, PR+, VDR+), UISO-BCA-4 (ER-, PR-, VDR+), and MDA-MB231 (ER-, PR-, VDR-). Cells were incubated for 7 days with increasing concentrations of 1α(OH)D5 in the range of 10-9M to 10-6M. There was no effect observed at 10-9M, and no cell toxicity was observed at the highest concentration. On the other hand, marked toxicity was observed when cells were incubated with 1,25(OH)2D3. These results suggest that cells required VDR in order to be responsive to either of the two vitamin D analogs. MDA-MB-231, which is characterized by the absence of ER, PR, and VDR, did not respond at all to any of the analogs. However, the presence of steroid receptors in these cells is not essential for the antiproliferative activities observed. For example, UISO-BCA-4, which lacks both ER and PR but expresses VDR, responded to both 1,25(OH)2D3 and 1α(OH)D5.

Table 1 below shows the p53, steroid, and vitamin D receptor status of the breast cancer cell lines used in these studies.

Table 1: p53, Steroid, and Vitamin D Receptor Status of Breast Cancer Cell Lines

Cell line	P53 status	ER status	PR status	Her-2 expression	VDR Status
MCF-7	wild	positive	positive	low	Positive
ZR-75-1	wild	positive	positive	medium	Positive
T-47D	mutant	positive	negative	-	Positive
BT-474	mutant	positive	positive	high	Positive
UISO-BCA-1	mutant	negative	negative	medium	Positive
MDA-MB-231	mutant	negative	negative	low	Negative
MDA-MB-468	mutant	negative	negative	Low	Negative
MDA-MB-435	mutant	negative	negative	Low	Negative
MAXF-401	mutant	negative	negative	Medium	N/A
UISO-BCA-4	mutant	negative	negative	low	Positive

6

These studies demonstrated that all cell lines expressing VDR (VDR-positive) are responsive to vitamin D analog and have induced cell differentiation (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73), which is characterized by increased casein and lipid expression in the cells. Furthermore, morphologically the cells exhibit signs of cell differentiation. Most importantly, breast cancer cells that are both ER- and PR-positive in addition to being VDR positive, exhibit not only differentiation but also apoptosis. The typical effects of 1αhydroxyvitamin D5 on the cell cycle of BT474 breast cancer cell line, which is positive for ER, PR, and VDR, is shown below in Figure 1.

Figure 1: Effects of D5 on the Cell Cycle in BT474 Cells

% G-1	70.3
% S	16.1
% G-2	13.6

| % G-1	83.3
% S	14.9
% G-2	1.3
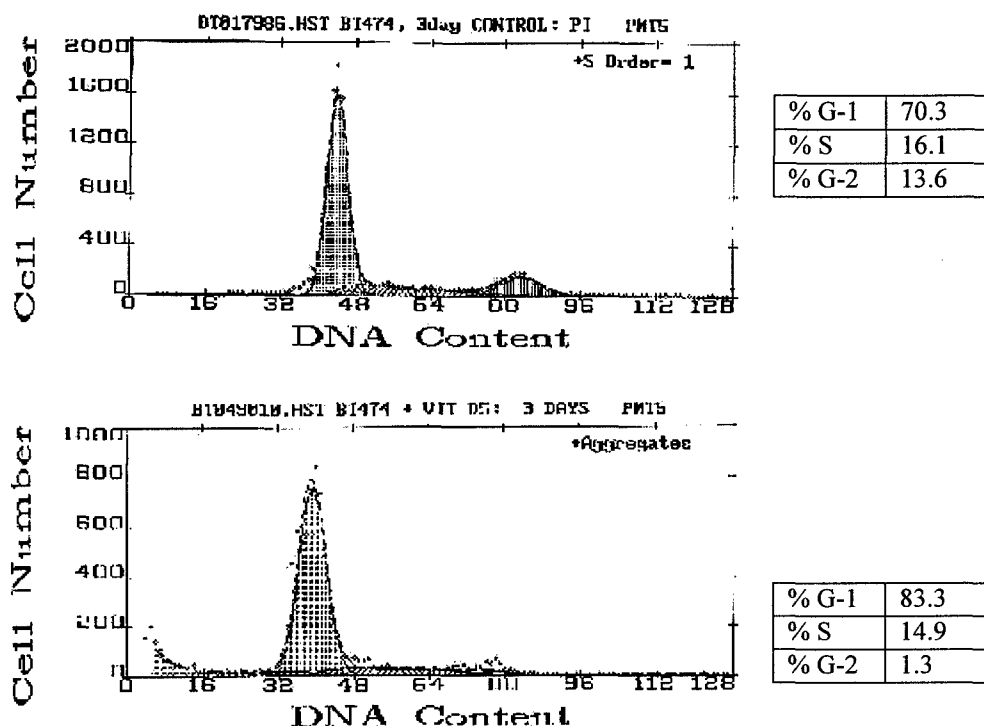
Selective effects of 1α-hydroxyvitamin D5 on transformed cells

A question was raised whether the effects observed against breast cancer cells can also be observed for normal breast epithelial cells. MCF12F cells are normal breast epithelial cells derived from a woman who did not have breast cancer. The cells were immortalized in culture and have been extensively used. These cells are commercially available, and this cell line was selected for the current study. Experiments were designed to compare effects of 1α(OH)D5 among these normal breast epithelial cells immortalized in culture, MCF12F, and DMBA-transformed MCF12F cells. The results are shown below in Figure 2 and Figure 3.

Figure 2: Effect of D5 on the Cell cycle of MCF12F Normal Human Mammary Epithelial Cells
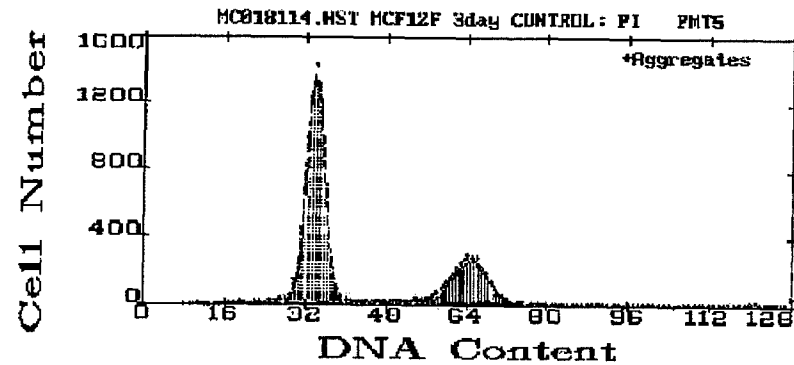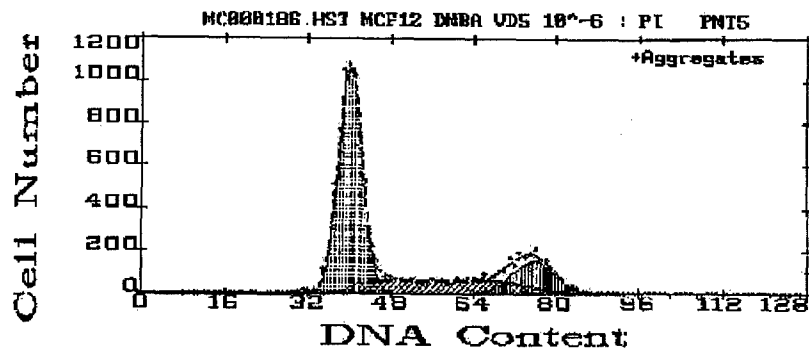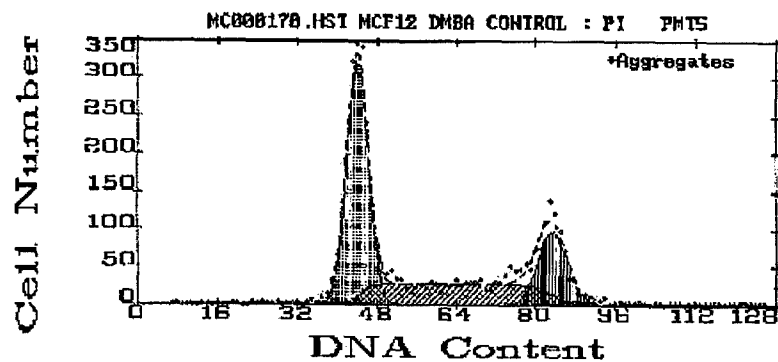

% G-1	66.6
% S	7.6
% G-2	25.6
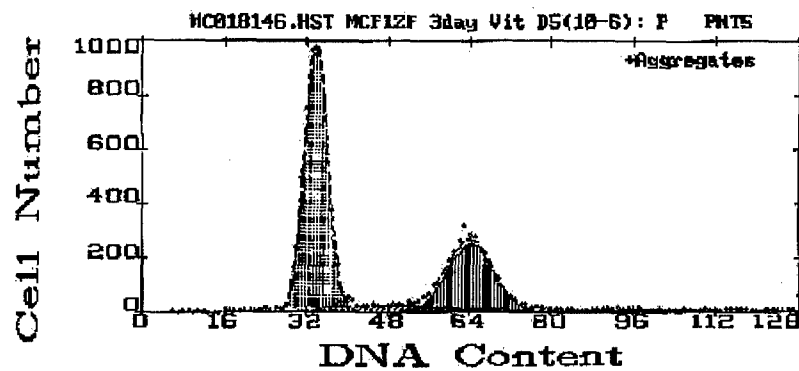
% G-1	65.1
% S	7.8
% G-2	27.1

Figure 3: Effect of D5 on Cell Cycle in Transformed MCF12FDMBA Cells

% G-1	49.6
% S	29.9
% G-2	20.5

% G-1	62.1
% S	21.9
% G-2	16.0

In order to further investigate this property, the MCF12F cells were transformed with chemical carcinogens N-methyl-N-nitrosourea (MNU) and 7,12 dimethylbenz(a)anthracene (DMBA). These carcinogen-treated transformed cells have altered growth rate. $1\alpha(OH)D5$ suppressed the proliferation of the carcinogen-treated cells, whereas the parent MCF12F cells did not respond (Figures 2 and 3). These results indicate that the effect of $1\alpha(OH)D5$ is selective for breast epithelial cells with altered growth characteristics as observed in breast cancer cells or carcinogen-induced transformed cells.

In summary, these results demonstrate that proliferation of BT474 cells, like the other VDR+ breast cancer cells, was inhibited by $1\alpha(OH)D5$. Moreover, cell cycle analysis showed that there was a G1 arrest in BT474 cells following exposure to 1 μM $1\alpha(OH)D5$ for 7 days. However, there was no antiproliferative effect or cell cycle arrest observed in non-transformed MCF12F cells.

Selectivity of efficacy of $1\alpha(OH)D5$ for breast cancer tissue and not normal mammary epithelium

Breast tissue samples obtained at the time of surgery of reduction mammoplasty and breast cancer samples were incubated with 1 μM $1\alpha(OH)D5$ for 72 hours. The tissues were fixed in formalin and tissue sections were prepared. The cell proliferative index was determined by staining tissue sections with Ki67. Results indicated that there was no effect of $1\alpha(OH)D5$ on the cell proliferation of the normal breast epithelial cells in culture whereas the Ki67 staining was considerably reduced in the cancer tissues (data not shown). These results are consistent with the results described in previous sections for MCF12F normal breast epithelial cells and normal mammary glands in organ cultures. These histopathologic results corroborate the findings on the growth-inhibitory effects of vitamin D5 in the in vitro studies described earlier.

Mechanism of Action of $1\alpha(OH)D5$

The effect of $1\alpha(OH)D5$ is mediated by inducing cell differentiation, and VDR is essential for the function

To examine this hypothesis, we determined the differentiating effects of 1α-hydroxyvitamin D5 in T47D human breast cancer cells. Cells incubated with either 10 or 100 nM of $1\alpha(OH)D5$ inhibited cell proliferation in a dose-dependent manner, as measured by the MTT assay. This inhibition in cell proliferation was comparable to that seen with 1,25-dihydroxyvitamin D3. Both vitamin D analogs induced cell differentiation, as determined by induction of casein expression and lipid production (Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6). Induction of cell differentiation is often correlated with inhibition of cell proliferation. Casein and lipid expression are characteristics of normal lactating mammary glands. Thus, induction of these differentiation markers suggests that the cancer cells are reverting to express normal function. Since the cell-differentiating effect of vitamin D is considered to be mediated via VDR, we examined the induction of VDR mRNA using RT-PCR. The results showed that, in T47D cells, both 1,25-dihydroxyvitamin D3 and 1-Hydroxyvitamin D5 induced VDR mRNA in a dose-dependent manner. Moreover, both analogs of vitamin D up-regulated expression of vitamin D Response Element (VDRE)-VDR interaction as determined by CAT reporter assay (Lazzaro G et al. Eur J Cancer. 2000 Apr;36(6):780-6). These results

collectively indicated that 1α-Hydroxyvitamin D5 may mediate its cell-differentiating action via VDR in a manner similar to that of 1,25-dihydroxy D3

The differentiation properties of 1α(OH)D5 were further investigated in breast cancer cells. Following 10 days treatment with 1α(OH)D5 (10-7 M in UISO-BCA-4), we observed induction of intracytoplasmic casein, intracytoplasmic lipid droplets, ICAM-1, nm23, and specific biomarkers associated with breast cancer cell differentiation. 1α(OH)D5 treatment also showed induction of vitamin D receptor and TGFβ1 proteins in the cells. These results, along with the ones described in previous sections, suggest that the action of 1α(OH)D5 is mediated by VDR in breast cancer cells.

1α(OH)D5 induces apoptosis in ER+, PR+, VDR+ breast cancer cells

In ER+, PR+, and VDR+ breast cancer cells, 1α(OH)D5 induces apoptosis as well as cell differentiation, but only cell differentiation in ER-, PR-, and VDR+ breast cancer cells. We further evaluated 1α(OH)D5-induced cell apoptosis in BT474 cells. Cell cycle analysis results indicated that, in BT474, the cell growth was arrested in G1 phase. Moreover, acridine orange/ethidium bromide staining showed apoptotic fragmentation of nuclei in these cells (Figure 4).

Figure 4: Induction of Apoptosis in BT474 Cells by 1α(OH)D5

| Control | VD310-7 | VD5 10-6 |
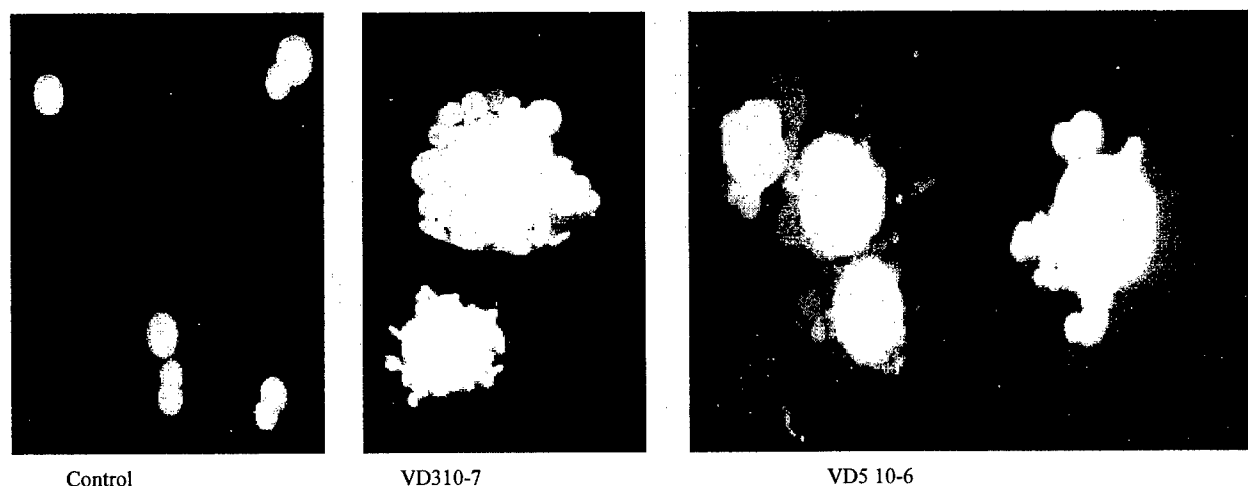
Since the only difference between these cells and BCA-4 cells was the presence of ER and PR in BT474 cells, we incubated BT474 cells with 10 nM estradiol for 5 days in steroid-stripped medium and examined estrogen-inducible expression of progesterone receptors. These cells require estradiol in the medium for cell proliferation and for the expression of estrogen-inducible genes such as progesterone receptors. The control cells expressed a higher intensity of progesterone receptors. 276 cells/351 were positively stained for PgR, whereas this PgR expression was down-regulated when the cells were incubated with 10 nM estradiol plus 1α(OH)D5.

The effect of 1α(OH)D5 was further determined by first determining the expression of D-altered genes by gene array analysis. Using the Unigene system, which examines a chip of 10,000 genes, mRNA prepared from 1α(OH)D5-treated cells was compared with that of control

cells. Results showed that progesterone receptors, PS2, trefoil factor, and 24-hydroxylase were some of the genes most altered by $1\alpha(OH)D5$. These results are shown below. They clearly indicate that the effect of $1\alpha(OH)D5$ in ER+ breast cancer cells is in part mediated by down-regulating estrogen-inducible genes (See Figure 5).

Figure 5: Selected Genes from Micro-Array Analysis of D5-Treated BT474 Cells Using Human UniGene 1 (10,000 genes)

Gene Name	Differential Expression (fold)	Statistical Significance
Estrogen-inducible Genes		$p < 0.01$
Trefoil Factor 1 (pS2)	5.7 ↓	$p < 0.01$
Trefoil Factor 3 (Intestinal)	3.5 ↓	$p < 0.01$
Progesterone Receptor	3.2 ↓	
Vitamin D Regulated Genes		
Vitamin D Receptor	1.1 ↑	NS
Cytochrome P450 (Vitamin D Hydroxylase)	6.3 ↑	$p < 0.01$
Differentiation-related Genes		
Cadherin 18 type 2	3.5 ↑	$p < 0.01$
Matrix Metalloproteinase 9 (type IV Collagenase)	1.5 ↓	$p < 0.05$
Laminin Receptor 1	1.9 ↓	$p < 0.01$
Apoptosis-related Genes		
Caspase 3 (Apoptosis-related Cysteine Protease)	1.7 ↑	$p < 0.01$
Cell Growth Related Genes		
Proliferating Cell Nuclear Antigen	1.2 ↓	NS
Thymidine Kinase 2 (Mitochondrial)	1.9 ↑	$p < 0.01$

Effects of pretreatment of breast cancer cells with $1\alpha(OH)D5$ on subsequent development of tumors in mice

These studies were conducted with VDR-positive UISO-BCA-4 cells developed in our laboratory. The experiment was divided into two groups. In one group, the cells were treated with 1 μg/ml $1\alpha(OH)D5$ for 10 days, and the other group served as controls. These cells were inoculated in athymic mice and allowed to grow. All five animals in the control group developed tumors, whereas there was only a scab-like lesion in the animals where the cells were pretreated with 1 μM $1\alpha(OH)D5$ prior to inoculation. Inhibition of growth and progression of tumor in this model was attributed to $1\alpha(OH)D5$-induced differentiation of treated cells. It can be interpreted that $1\alpha(OH)D5$-induced differentiation in turn inhibited the growth and progression of breast cancer (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73).

Tissue distribution of 1α-hydroxyvitamin D5

Tissue distribution studies have not been carried out in depth due to the unavailability of radioactive $1\alpha(OH)D5$. We are currently in the process of having radioactive $1\alpha(OH)D5$ synthesized. Preliminary studies were carried out to determine if $1\alpha(OH)D5$ can be recovered

from plasma, liver, and mammary tumors after 2 months of feeding with 12.5 µg/kg diet 1α(OH)D5 in mice. The tissues were pulverized and extracted with methanol, and vitamin D metabolites were separated on a reversed-phase HPLC column. The HPLC profile showed the presence of 1α(OH)D5 parent compound in both mammary tissues and liver. There was no peak coeluting with 1,25 dihydroxyvitamin D3. The metabolites have not been identified due to the unavailability of standards needed for identification. However, the HPLC profile showed no peak coeluting with 1,25 dihydroxyvitamin D3.

Effects of 1α-Hydroxyvitamin D5 on the in vivo growth of breast cancer cells in athymic mice

Previously, we reported that breast cancer cells grow more efficiently in athymic mice when they are mixed 1:1 v/v of Matrigel (BD Biosciences, Palo Alto, CA) (Mehta RR et al. Breast Cancer Res Treat. 1993;25(1):65-71). Subcutaneous injection of 1-2 million cells into athymic mice results in the development of breast tumor. Histopathologically, these tumors are comparable to the parent cancers. We evaluated the effects of dietary modulation with 1α(OH)D5 on the development of breast cancers of several breast cancer cell lines. These include ZR75-1, T47 D, UISO BCA-4, and MCF-7. In most of the studies, five animals per group were used. The control animals received vehicle-containing diets, whereas the powdered diet was mixed with either 10 or 20 µg/kg of diet of 1α(OH)D5. The mice started receiving experimental diet one day after inoculation of cancer cells. The tumor size was monitored by measuring with vernier calipers. The experiment was terminated either 60 days post inoculation with tumor cells or if the tumors reached a large size (>2 cm). Results showed that 1α(OH)D5 suppressed the growth of breast cancer cells in athymic mice in most experiments except for MDA-MB-231 cells, which did not express VDR (9). An example of growth suppression of BT474 cells in athymic mice is shown in Figure 6: Growth of Breast Cancer Cells in Athymic Mice. The results collectively suggest that 1α(OH)D5 has a growth inhibitory role in VDR+ human breast cancer.

Figure 6: Growth of Breast Cancer Cells in Athymic Mice
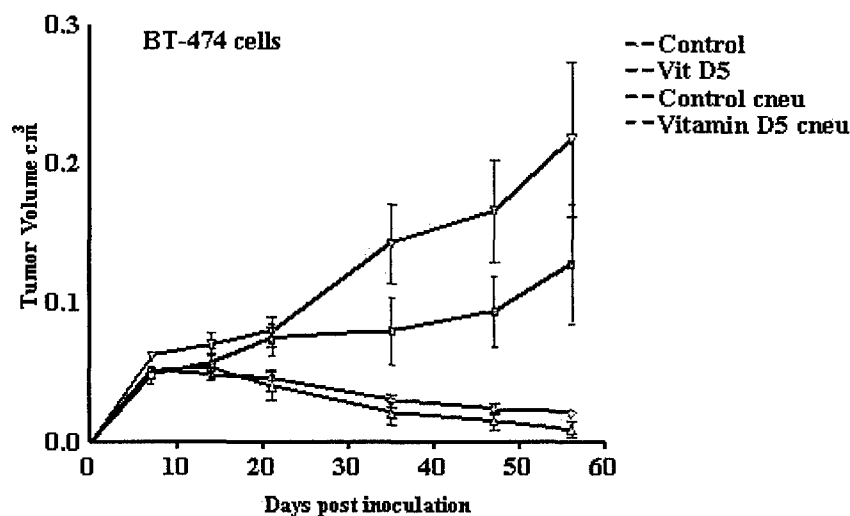
The role VDR plays in therapeutic efficacy in human breast cancer: UISO-BCA-4 cells developed in our laboratory were divided into two groups. One group of cells was treated with 1 μg/ml 1α(OH)D5 for 10 days, and the other group served as controls. These cells were inoculated in athymic mice and allowed to grow. All five animals in the control group developed tumors, whereas there was only a scab-like lesion in the animals where the cells were pretreated with 1 μM 1α(OH)D5 prior to inoculation. These findings can be interpreted as showing that 1α(OH)D5 induced differentiation, which in turn inhibited the growth and progression of breast cancer (Mehta RR et al. Int J Oncol. 2000 Jan;16(1):65-73).

Toxicology

The main reason new analogs of vitamin D are being developed is to generate compounds with reduced or absent toxicity. The analog 1α(OH)D5 is one such relatively non-toxic vitamin D analog. We have completed an extensive series of preclinical toxicity studies for this vitamin D analog. In this section, we describe gross toxicity, calcemic activity in vitamin D-deficient rats, and preclinical toxicity studies under GLP in two species: rats and dogs.

Gross toxicity

Treatment of animals with vitamin D analogs often results in loss of body weight gain. This is the first noticeable toxicity. As shown below, the maximum tolerated doses were determined for athymic mice, Balb/c mice, and Sprague-Dawley rats. These doses represent concentrations at which there was no loss of body weight gains and no adverse effects on general health. Lethargy, loss of body fur, loss of weight, or loss of gain of body weight are considered as signs of gross toxicity. The animals were weighed twice a week and observed daily for lethargy and other noticeable changes. However, no apparent side effects were noticed as a result of 1α(OH)D5 feeding in these animals.

Experiments were carried out to determine maximum tolerated dietary dose of 1α(OH)D5 for rats. Sprague Dawley rats were separated into 11 groups of 10 animals each. Group 1 served as a control. Rats in other groups received either five doses (0.8, 1.6, 3.2, 6.4 and 12.8 g/kg) of 1,25(OH)2D3 or five doses (3.2, 6.4, 12.5, 25 and 50g/kg) of 1α(OH)D5 for six weeks. Results showed that there was hypercalcemia and loss of body weight observed at 12.8 g/kg diet, whereas there was in fact increased body weight observed at 50g/kg of 1α(OH)D5 dose level. In a separate study there was no adverse effect of D5 on the body weight gain was observed at 100g/kg diet. Therefore, 1α(OH)D5 can be tolerated at a much higher concentration than the dihydroxy-D3 analog of vitamin D.

Measurements of calcemic activity in vitamin D-deficient rats

Male rats three weeks of age were fed diet containing 0.47g% calcium, 0.3g% phosphorus, and free of vitamin D. After three weeks of consumption of this diet, serum calcium levels were measured on selected animals. Animals exhibiting serum calcium values of less than 6.0 mg/dL were considered as vitamin D-deficient. The rats were treated intragastrically with appropriate vitamin D analog for 14 days. At the end of the study, the calcium concentrations were measured in the serum. The vehicle-treated control rats showed calcium concentrations of 5.4+ 0.3 mg/dL (mean + standard deviation). When animals were injected with 0.042 μg/kg/day of vitamin D analogs, plasma calcium concentrations of 6.0+0.6 mg /dL for 1α(OH)D5 were observed (11% increase over control, not significant from that of the control) and 8.1+ 0.1 mg/dL

for $1\alpha,25(OH)D2$ D3 (50% increase over control, significant increase). A higher concentration of 0.25 µg/kg/day of $1\alpha(OH)D5$ exhibited a plasma calcium concentration of 8.1± 0.1 mg/dL as compared to 10.1± 1.8 for $1\alpha(OH)2D3$. Although both analogs increased serum calcium in comparison to the control samples, these results showed overall lower calcemic effects induced by $1\alpha(OH)D5$ as compared to $1\alpha,25(OH)2D3$. At higher concentrations of $1\alpha,25(OH)2D3$, there was an 87% increase in the plasma concentration of calcium as compared to the vehicle-treated rats. In contrast, when animals were injected with a higher concentration of $1\alpha(OH)D5$, there was only a 50% increase in the plasma calcium concentration as compared to controls.

Preclinical Toxicity (GLP)

Four-week oral (gavage) toxicity studies were performed on rats and dogs at the IIT Research Institute in accordance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice (GLP) regulations as set forth in the Code of Federal Regulations (21 CFR Part 58). Copies of the entire document(s) for both rats and dogs experiments are available upon request; this topic was also covered in detail in the 2002 Annual Report for this study.

Synthesis of 1α-Hydroxyvitamin D5 under GMP

As proposed in the original application the synthesis of 1α-Hydroxyvitamin D5 is being carried out by Drs. Robert Moriarty and Raju Penmasta at Conquest Inc. (formerly known as Steroids Ltd.). Dr. Moriarty has synthesized and supplied 1α-hydroxyvitamin D5 for all our prior studies. As a part of this project, a subcontract to Dr. Moriarty is awarded for him to supply 1 gram of the compound for preclinical toxicity and 1 gram of the analog synthesized under Good Manufacturing Practice (GMP). Dr. Moriarty already synthesized and supplied 1 gram of $1\alpha(OH)D5$ for preclinical toxicity. Experiments described under preclinical toxicity used this newly synthesized compound. The synthesis of D5-analog under GMP is completed. The compound is secured in UIC Pharmacy under close control of Ms Bressler, a registered Pharmacist.

Plan for the Clinical Trial

We have submitted an application to the FDA (IND #56509) for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA has requested a stability study of 1a(OH)D5 under GMP/GLP conditions. We have given a contract to an FDA-approved laboratory to provide us with this data. It appears that once the stability data is received, the FDA will approve our application. The UIC IRB has already approved the clinical protocol. Following FDA approval, we will provide all the necessary papers to the DOD committee on human experimentation. It is expected that a "go ahead" will be received from DOD and the clinical trial will be initiated.

Key Research Accomplishments

Nonclinical studies:

Studies in human tumor/normal breast tissues:

The effects of in vitro 1α(OH)D5 were observed in normal breast tissues, fibroadenomas, and breast carcinomas obtained from women with confirmed diagnosis of the disease. Our results show that:

♦ Normal breast tissue retains the original alveolar and ductal structures when incubated in the culture medium used in this study. Breast epithelial cells appear to be normal and alive for 72 hours. All epithelial cells show VDR expression. Many appear to be proliferating, as evident from Ki-67 staining. 1α(OH)D5 (1 μM) treatment shows no toxic effect on the breast epithelial cells; all alveolar and ductal structures are preserved. 1α(OH)D5 has no effect on cell proliferation.

♦ Breast fibroadenomas retain normal structures in in vitro culture for 72 hours. Following incubation with 1α(OH)D5, many alveolar structures show apoptotic or degenerative epithelial cells. The cells unaffected by 1α(OH)D5 show high expression of VDR.

♦ Breast carcinomas treated with 1α(OH)D5 show a significant number of cells undergoing pyknosis or apoptosis.

Studies using established human breast carcinoma cell lines:

♦ Our results on competitive binding studies with VDR indicate that 1α(OH)D5 has relatively lower binding affinity than 1,25(OH)2D5. These results suggest that 1α(OH)D5 may possibly mediate its cell-differentiating and antiproliferative actions through VDR and also through other pathways.

♦ We established 4 different cell lines with different VDR and ER status. These cell lines were cloned and used to determine interaction between ER and VDR and the effect of 1α(OH)D5 on these cells. The growth inhibitory effects of 1α(OH)D5 were observed in Vitamin D receptor positive (VDR(+)) breast cancer cells, but not in highly metastatic VDR(-) breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D5 action may be mediated, in part, by VDR. Breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER- cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ vs. ER- cells.

♦ Studies on MDA-MB-231 (ER+, VDR+) cells clearly indicate that 1α(OH)D5 influences ER expression in breast cancer cells.

♦ We have further confirmed our previous findings that 1α(OH)D5 inhibits proliferation and induces cell differentiation markers in breast tumors (tumors obtained from patients) in vitro.

Preclinical Toxicity Studies:

♦ We have completed the preclinical toxicity study in male and female rats under GLP. Males and females were given 1-10 μg/kg body weight 1α(OH)D5 by oral gavage for 28 consecutive days. 1α(OH)D5 showed no serious toxic effect. No animals died during the study, and no adverse treatment-related clinical signs of toxicity were observed. No treatment-related effects on body weight, weekly or total body weight gain, or food consumption were observed during the study. Increased serum calcium levels in both sexes at the high dose level and in females at the mid dose level. Microscopic lesions consisting primarily of increased renal mineralization were seen in males at the mid and high dose levels and in females at all dose levels. Although a no-effect level was not established in this study, the toxicological significance of microscopic lesions occurring at all dose levels was considered to be minimal because of the minimal severity of the lesions and because these lesions also occur as incidental findings in rodent studies. The effect of 1α(OH)D5 was reversible. Within two weeks after discontinuation of the treatment, serum calcium levels and renal mineralization lesions reached the same levels as the control group.

♦ We have completed preclinical toxicity studies in dogs under GMP. 1α(OH)D5 was tested (5-45/90 μg per kg body weight dose). The compound was given to animals daily by gavage for 28 days. At 5 μg/kg body weight dose, hypercalcemic activity was detected. The compound had some drug-related toxicity at 5 μg/kg body weight dose. All higher doses tested were toxic and hypercalcemic in dogs. Although we observed drug-related toxicity in our preclinical toxicity studies, doses tested were significantly higher than those proposed for the phase I clinical trial.

Phase I and Phase II clinical trials:

♦ We have prepared sufficient quantity of 1α(OH)D5 under GMP for future clinical studies. According to the requirement of FDA, we have secured the compound at the UIC pharmacy under direct control of Ms. Linda Bressler, Registered Pharmacist at UIC Hospital.

♦ We have submitted an application to the FDA (IND #56509) for approval for the initiation of a Phase I/II clinical trial of this analog in metastatic breast cancer. The FDA has requested a stability study of 1α(OH)D5 under GMP/GLP conditions. We have given a contract to an FDA-approved laboratory to provide us with this data. It appears that once the stability data is received, the FDA will approve our application. The UIC IRB has already approved the clinical protocol and informed consent form.

Tasks originally proposed but not completed in the proposed time line and are currently under investigation:

1. Initiation of a phase I clinical trial of 1α(OH)D5 was originally proposed to initiate by 2001; however, due to FDA and UIC IRB hold, it was delayed. The UIC IRB approval has been obtained. The FDA wishes further studies on the stability of Vitamin D5. Vitamin D analog is synthesized under GMP conditions. We are doing these studies, and as soon as the results are available they will be sent to the FDA.
   We hope that in the near future patient accrual will be initiated.

2. As soon as FDA approval is received, we plan to initiate a phase I trial. The vitamin D analog is synthesized under GMP regulations and is available for the clinical use.

3. We have identified genes that are regulated by $1\alpha(OH)D5$. Many of these genes are those regulated by estrogens. We are currently evaluating how $1\alpha(OH)D5$ modulates expression of various genes.

4. Radioactive $1\alpha(OH)D5$ is synthesized by a commercial company. We will perform pharmacokinetics study of the compound as soon as the radioactive compound is available.

Reportable outcomes

Publications:

1. Lazzaro G., Agadir A., Qing W., Poria M., Mehta R.R., Moriarty R.M., Zhang X., Mehta R.G. Induction of differentiation by $1\alpha(OH)D5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur. J. Cancer, 36, 780-786, 2000

2. Mehta R.R., Mehta R.G. Differentiation of human breast carcinoma cell line by a novel vitamin D analog: $1\alpha(OH)D5$. Int J Oncology 16: 65-73, 2000.

3. Lazzaro G., Agadir A., Qing W., Poria M., Mehta R R. Moriarty R.M., Zhang X, Mehta R.G. Induction of differentiation by $1\alpha(OH)D5$ in T47D human breast cancer cells and its interaction with vitamin D receptor. Eur J Cancer 36: 780-786, 2000.

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6. Mehta RG, Hussain EA, Mehta RR, Das Gupta TK. Chemoprevention of mammary carcinogenesis by 1alpha-hydroxyvitamin D5, a synthetic analog of Vitamin D. Mutat Res. 2003 Feb-Mar;523-524:253-64.

7. Punj V, Graves JM, Mehta RR. Effect of vitamin D analog (1alpha hydroxy D5) immunoconjugated to Her-2 antibody on breast cancer. Int J Cancer. 2004 Mar 1;108(6):922-9.

Presentations at national and international meetings:

8. Mehta R.R., Mehta R.G., Hussain E., Moriarty R., Mehta R.R. and Das Gupta T.K. Chemoprevention of mammary carcinogenesis by synthetic analog of vitamin D. Mutation Res. Seoul, Korea, 2002.

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11. Hussain E.A., Bhat K., Mehta R.R. and Mehta R.G. $1\alpha(OH)D5$ induces apoptosis and cell cycle arrest in BT-474 breast cancer cells. Proc Am Assoc Cancer Res 42: 209, 2001.

Conclusions

We have completed the tasks originally proposed in the application. We have performed studies in cell lines and have completed detailed preclinical toxicity studies in dogs and rats under GLP. We have completed synthesis of $1\alpha(OH)D5$ under GMP for future clinical trial. In vitro studies in clinical specimens obtained from women suggest that $1\alpha(OH)D5$ has no effect on normal breast tissues; it inhibits cell proliferation in tumor cells. This implies that it has no bad effects on normal breast tissues but does inhibit cancer growth. $1\alpha(OH)D5$ or its active metabolite possibly interacts with estrogen receptor. We have submitted our IND application to the FDA, and are currently conducting stability studies requested prior to IND approval. The UIC IRB has approved the clinical protocol and informed consent form for the Phase I clinical trial.

Our findings to date imply that $1\alpha(OH)D5$ has no bad effects on an overall biologic system (beagle dog and rats) or on normal breast tissues but does inhibit cancer cell growth. The fact that we are applying for approval to bring a vitamin derivative to clinical trial represents a very hopeful development in cancer treatment.

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Appendices

Appendix 1   Protocol for Clinical Trial

Appendix 2   Publications

                   Publication 1

                   Publication 2

                   Publication 3

Appendix 1: Protocol for Clinical Trial

A Phase I/II Trial of 1αhydroxyvitamin D₅ in the Treatment of

Metastatic Breast Cancer

Co-Investigators

Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

George I. Salti, M.D.

Sub-Investigators

Cathleen Schaeffer, R.N., B.S.N., O.C.N.

Linda Bressler, Pharm.D.

Protocol No.: UISO – D5 – 001 – 03

Protocol Synopsis

Title: A Phase I/II Trial of 1αhydroxyvitamin D_5 in the Treatment of Metastatic Breast Cancer

Objective: To evaluate the safety and efficacy of 1αhydroxyvitamin D_5 (Vitamin D5) in the treatment of patients with metastatic breast cancer

Population: Patients with metastatic breast cancer

Sample Size: 42 patients

Dosage/Treatment: Based on completed preliminary studies (see attached investigator's brochure), the first six (6) patients will receive a single daily oral dose of 1αhydroxyvitamin D_5 starting at 5 μg gelatin capsule. If there is no toxicity (see Section 8.0), the next 6 patients will be treated similarly with 10 μg daily. The dose will be escalated (in 5 μg increments) up to a maximum of 35 μg daily.

Duration: Treatment will be continued for three months (12 weeks) and/or disease progression, though blood tests will continue monthly for 28 weeks and then every two months for an additional six (6) months of follow-up.

Endpoints: Safety – Clinical and laboratory adverse reactions will be closely monitored by periodic physical and laboratory examination.

Grade 3 nonhematologic or grade 4 hematologic toxicity will define the maximum tolerated dose (MTD) (Appendices 1 and 2). Evidence of hypercalcemia will be the primary determining factor in dose escalation.

Efficacy - Clinical response as measured by decrease in measurable disease determined by physical examination, radiographic studies, and/or nuclear medicine scans.

Co-Investigators: Tapas K. Das Gupta, M.D., Ph.D., D.Sc.

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Sub-Investigators:

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1.0 OBJECTIVES

To evaluate the safety and chemotherapeutic efficacy of 1αhydroxyvitamin D_5 (1α(OH)D_5) in patients with metastatic breast cancer.

2.0 BACKGROUND AND RATIONALE

2.1 Disease background

Although a number of patients with localized breast cancer can be adequately treated with surgery and radiation therapy, for the vast majority of patients additional use of conventional chemotherapeutic agents and hormonal therapy is necessary. While initially responsive to various cytotoxic and hormonal modalities, most breast cancers ultimately acquire resistance to current systemic therapies. Thus, the development of effective new therapeutic modalities is critical. Recently, several vitamins and vitamin analogs have been the foci of investigation as therapeutic agents for various malignancies. Among various vitamins, vitamin A[1,2,3,4,5,6] and vitamin D[7,8,9,10,11,12] have shown the most promising results. The active metabolite of vitamin D_3, 1α,25 dihydroxy D_3 (1α,25(OH)2D_3), has been conclusively shown to induce differentiation *in vitro* in a variety of cancers, including breast cancer.[13,14,15,16,17,18,19,20] Similarly, physiologic levels of 1α,25 dihydroxyvitamin D_2, which has less calcemic activity, have been shown to induce differentiation in androgen-independent prostate cancer. However, most of the D group of vitamins and closely related metabolites are limited in their translational use due to their hypercalcemic activity. Numerous analogs of vitamin D_3 have been synthesized in search of a non-calcemic or relatively less calcemic vitamin D with similar antiproliferative effects.[21,22,23,24,25,26,27]

In the present study, we aim to evaluate the chemotherapeutic potential of another analog: 1α(OH)D_5. Using breast tumors with different histologic subtypes and with different molecular and biological characteristics, the effects of 1α(OH)D_5 (D_5) on breast cancer cell growth and differentiation both in vitro and in the athymic mouse model have been evaluated (for details, see investigator's brochure). Based on these laboratory studies, the present phase I/II clinical trial will be initiated, to determine the dose tolerance and efficacy of 1α(OH)D_5 in advanced breast cancer patients.

2.2 Vitamin D_5 Analog Background

1α(OH) D_5, a new non-calcemic vitamin D analog synthesized by our group, has shown potent activity against human breast cancer in xenograft models.[28] The analog is 10 times less calcemic than 1α,25(OH)D3, when evaluated in vitamin D-deficient rats. It inhibited carcinogen-induced pre-cancerous lesions in mouse mammary gland organ culture. It also inhibited growth of ER+ MCF-7, ZR-75, and T47 D cells, and ER- UISO-BCA-4 human breast cancer cells in culture. The ability to induce

differentiation was evaluated in detail in ZR-75 and UISO-BCA-4 cells. The cells treated with 10^{-8}M $1\alpha(OH)D_5$ displayed altered cellular organization, resulting in the formation of ductlike structures in culture dishes. This phenomenon was accompanied by up-regulation of casein, ICAM, and nm23 expression.[29] Expression of these proteins is correlated with differentiation of breast cancer cells. Moreover, the differentiated cells (i.e., cells treated with D_5 analog) did not form adenocarcinoma in athymic mice as compared to the development of tumors in 100% of the control group. In addition, dietary supplementation of $1\alpha(OH)D_5$ inhibited the growth of breast cancer cells transplanted into mice.[29]

The mechanism of action of vitamin D is poorly understood.[8] In our studies with ER-positive and ER-negative breast cancer cells, $1\alpha(OH)D_5$ exerted relatively more cytostatic and cytotoxic effects on ER-positive cells. The reason for this differential effect is currently being pursued in our laboratory. At present, it is suggested that vitamin D induces its own receptors (i.e., vitamin D receptors [VDR]). We have shown that both $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$ induce VDR in breast carcinoma cell lines, as determined by immunocytochemistry, western blot analysis and RT-PCR.[29,30] Furthermore, the induction of VDR was accompanied by increased expression of $TGF\beta1$ and $TGF\beta2$. We hypothesize that $1\alpha(OH)D_5$, when administered to women with breast cancer or women with premalignant lesions, will induce differentiation of dedifferentiated and/or premalignant cells and thus will prevent progression and/or development of malignancy.

2.3 Pharmaceutical and chemical data

In order to synthesize 1α-hydroxyvitamin D_5, vitamin D_5 (24 ethyl vitamin D_5) was first synthesized from β-sitosterol. Vitamin D_5 was converted to 1α-hydroxyvitamin D_5 by following the Paaren-DeLuca hydroxylation sequence. The compound was crystallized and characterized by $_1$H NMR, mass spectroscopy, and UV and IR spectroscopy. The purity was checked by HPLC analysis. These results have previously been described in detail.[28] The structure of 1α-hydroxy-24, ethyl cholecalciferole ($1\alpha(OH)D_5$) is shown in Appendix 3.

Preclinical studies, including cell culture experiments, pharmacokinetics, toxicity, and mechanism of action experiments, have been performed with crystalline compound; whereas, for the clinical trial, the crystalline compound will be prepared in gelatin capsules.

Dosage form preparation will be performed at the University of Illinois Hospital Pharmacy (Appendix 4 includes complete protocol for final dosage form preparation). To provide sufficient bulk to encapsulate the required microgram level dosage of study medications the crystalline 1α-hydroxyvitamin D_5 will be dissolved in alcohol and serial dilutions

performed to obtain appropriate concentrations. The 1α-hydroxyvitamin D_5 solution will then be absorbed into a suitable carrier (corn starch). After the alcohol is dried off and the material mixed, suitable quantities will be encapsulated. Study medications will be prepared on a weekly basis producing sufficient quantities of medication (42 capsules) to treat the six patients in the dosing cohort for one week. Additional capsules will be produced in every batch for content uniformity studies and periodic stability analysis.

3.0   STUDY HYPOTHESIS

We hypothesize that $1\alpha(OH)D_5$, when administered to women with breast cancer, will induce differentiation of dedifferentiated malignant cells and thereby prevent progression of malignancy. This is a phase I/II, single site, single arm, dose-escalation study.

4.0   OBJECTIVES

4.1   To determine the toxicity of $1\alpha(OH)D5$ in humans.

4.2   To obtain preliminary data on the efficacy of $1\alpha(OH)D5$ in advanced breast cancer patients.

5.0   DOSE AND ROUTE OF ADMINISTRATION

The present dosage schedule has been calculated based on our own preclinical data and recently reported Phase I trials with D_3 analogs for prostatic cancer.[31,32,33]

The major dose-limiting toxicity of vitamin D analogs has been hypercalcemia. Experimental evidence in animal studies indicates that the active metabolite was hypercalcemic at 2.99 nmole/kg body weight as compared to another analog in clinical trial EB1089 (seocalcitol), which was hypercalcemic at 5.5 nmole/kg body weight (BW). On the other hand, in preclinical toxicity studies we observed that $1\alpha(OH)D_5$ was non-calcemic at 11.65 nmole/kg BW. At 23.3 nmole/kg BW (10 μg/kg BW), there was an insignificant increase in calcium. As compared to 11.0 mg/dL in control rats, there was an 11.6 mg/dL in the 10-μg dose level.

Two Phase I/II clinical trials have been reported recently. In a calcitriol Phase I trial, 36 patients were given doses ranging from 2 to 10 μg every other day (QOD). At the highest dose, 3 out of 3 patients had hypercalcemia, whereas hypercalciurea was observed at all doses. No other toxicity was observed. The report concluded that calcitriol could be administered with tolerable toxicity.[31]

Another Phase II study was recently reported for advanced pancreatic cancer patients. In this study, 36 patients with advanced pancreatic cancer received once daily oral dose of seocalcitol (EB1089) with dose escalation every two weeks until hypercalcemia occurred. Once hypercalcemia occurred, the patients

were continued on maintenance therapy. The authors concluded that most patients tolerated 10-15 μg/day in a chronic treatment. Fourteen patients completed 8 weeks of treatment, whereas 22 patients were withdrawn due to clinical deterioration as a result of disease progression.[33]

Based on the experimental results indicating that $1\alpha(OH)D_5$ can be tolerated at a much higher concentration than other vitamin D analogs and the clinical studies described above, we expect no toxicity or hypercalcemia as a result of the proposed escalation protocol. Our protocol is designed with a starting dose of 5 μg/day (for a 70 kg person, this equals 0.035 μg/kg BW, with the highest dose of 35 μg/day/person (0.40 μg/kg BW). We do not anticipate that toxicity will be observed at these doses.

5.1 In the first group of six patients, a single, daily, oral dose of 1αhydroxyvitamin D_5 (5 μg/day) will be administered. Patients will be observed for signs and symptoms of hypercalcemia (see below for details). In the absence of any evidence of hypercalcemia, the $1\alpha(OH)D_5$ will be continued for 12 weeks (follow-up monthly blood tests will continue until week 28 with long-term follow-up testing to continue every two months for an additional six months or until death). If no toxicity is noted, the dose will be escalated in 5 μg increments, up to a total of 35 μg daily for 12 weeks (see Dose Escalation Schema below). In each of the seven dosing groups, six patients will be studied. There will be no dose escalation within the same cohort.

The following table shows the dose, route of administration, and escalation scheme. Between each dosing period listed below, there will be a minimum period of one week (7 days) during which results of the preceding dosing will be evaluated for safety parameters prior to initiating the next higher dosing level (see Section 5.3).

Dose Escalation Schema

Dose Level 1   Six(6) patients will be treated with oral administration of 5 μg/day for 12 weeks (84 days)

Dose Level 2   Six (6) patients will be treated with oral administration of 10 μg/day for 12 weeks (84 days)

Dose Level 3   Six (6) patients will be treated with oral administration of 15 μg/day for 12 weeks (84 days)

Dose Level 4   Six (6) patients will be treated with oral administration of 20 μg/day for 12 weeks (84 days)

Dose Level 5   Six (6) patients will be treated with oral administration of 25 μg/day for 12 weeks (84 days)

Dose Level 6   Six (6) patients will be treated with oral administration of 30 μg/day for 12 weeks (84 days)

Dose Level 7   Six (6) patients will be treated with oral administration of 35 μg/day for 12 weeks (84 days)

5.2 Duration of Treatment

5.2.1 Patients will be treated for a period of 12 weeks (follow-up blood tests will occur monthly until week 28 with additional follow-up tests every two months for an additional six months or until death).

5.2.2 It is estimated that the total number of evaluable patients will be entered within 24/36 months of the initiation of the study.

5.2.3 Patients demonstrating a progression of their disease as determined by the principal investigators will have their treatment discontinued and will be removed from the study. However, they will be followed for the 28 weeks of monthly blood tests (plus six months of blood tests at two month intervals or until death) for toxicity analysis.

5.2.4 An adequate trial requires three (3) months of treatment. An attempt will be made to keep patients on the study for the full three (3) months.

5.3 Dose Reduction and Stopping Criteria

5.3.1 Therapy may be discontinued at any time due to the development of any unacceptable toxicity.

If Grade 3 or higher non-hematologic or a Grade 4 hematologic toxicity develops, the study medication will be discontinued until recovery from toxicity. After complete recovery, the 1αhydroxyvitamin D_5 may be restarted at a dose of approximately one-half (minimum dose 5 μg/day) the original dose. Dosage reductions will be according to the following schedule:

Dose Cohort	Reduced Dose
5 μg/day	0 μg/day
10 μg/day	5 μg/day
15 μg/day	5 μg/day
20 μg/day	10 μg/day
25 μg/day	15 μg/day
30 μg/day	15 μg/day
35 μg/day	20 μg/day

5.3.2 If a Grade 3 or higher non-hematologic or a Grade 4 hematologic toxicity develops at the reduced dose level, the treatment will be discontinued and the patient will be removed from the study.

5.3.3 If two (2) out of six (6) patients develop Grade 3 non-hematologic or Grade 4 hematologic toxicity at the same dose level, no additional patient will be treated, and the study will be terminated.

6.0 PATIENT ELIGIBILITY

Forty-two (42) patients with metastatic breast cancer will be entered into this study. Patients with both estrogen receptor-positive and estrogen receptor-negative tumors will be eligible.

6.1 Inclusion criteria

6.1.1 Patients must have had histologically documented evidence of breast carcinoma.

6.1.2 Patients **must** have distant metastases (except brain metastases) and have a life expectancy of at least 3 months.

6.1.3 Patients must have failed at least one prior course of conventional treatment and must not be candidates for further treatment with anthracycline- or taxane-based therapy.

6.1.4 Patients must have signed an informed consent.

6.1.5 This study is confined to adult females age 18 or older.

6.1.6 ECOG Performance Status 0, 1, or 2 (see Appendix 1).

6.1.7 Patients must have no medical problems related to the malignancy that would pose an undue risk or that would limit full compliance with the study.

6.1.8 A minimum of 4 weeks must have elapsed since the completion of prior therapy, including hormonal therapy, chemotherapy, or radiation therapy, and patients must have fully recovered from such treatments.

6.1.9 Adequate baseline organ function as assessed by the following laboratory values within 30 days prior to study entry (with exception of corrected serum calcium and phosphorus, which are completed within seven days of study entry):

- Granulocyte count >1,500/mm^3, hematocrit >30%, and platelets >100,000/mm^3.

- Adequate renal function with estimated creatinine clearance >50 ml/min and or serum creatinine 2.5 or less.

- Corrected serum calcium level must be in the normal range (8.6-10.6 mg/dl) within seven days of study entry.

- No evidence of renal stones as determined by ultrasound of kidneys.

- Adequate liver function with SGOT, SGPT, LDH, and alkaline phosphatase <5x the upper limit of normal.

- PT and PTT not more than 1.5 times the upper limit of normal.

- bilirubin <2.0 mg/dl.

6.2 Exclusion criteria

6.2.1 Patients who are undergoing therapy with hormonal agents, cytotoxic agents, or any other therapy other than specified in this protocol. Concurrent focal radiation therapy with short-term supplemental steroids for spinal cord compression and/or severe bone pain unrelieved with standard pain medications is allowed.

6.2.2 Patients with brain metastases.

6.2.3 Patients with serious current illness, including untreated active infection.

6.2.4 Patients with any underlying conditions that would contraindicate therapy with study treatment (or allergies to D_5 used in this study).

6.2.5 Patients with prior or concomitant malignancies (except adequately treated basal cell carcinomas of the skin).

6.2.6 Patients with any other serious medical or psychiatric illness that would prevent informed consent.

6.2.7 Patients with breast cancer-related hypercalcemia (i.e., corrected serum calcium level outside the normal range of 8.6-10.6).

6.2.8 Patients with history of hypervitaminosis.

6.2.9 Patients who are either pregnant or lactating (all patients of childbearing potential will receive a pregnancy test within 7 days of study initiation).

6.3 Concomitant medication and treatment

All medications or treatments should be recorded. All questions regarding concomitant medications will be referred to the study investigators.

<u>Medications and treatment not allowed</u>

The following drugs and therapies are EXCLUDED while the patient is on study medication:

- Hormonal therapy, including steroids (However, patients are eligible to enroll if they are diabetic requiring insulin or if they are taking steroids as an adjunct to focal radiation therapy in cases of spinal cord compression and/or severe bone pain.)

- Chemotherapy

- Radiation therapy other than what is allowed (see 6.2.1)

- Megadose vitamin therapy

- Systemic therapy for hypercalcemia or biphosphinate treatment for any other therapy.

7.0 PATIENT EVALUATIONS

7.1 Pretreatment screening and baseline evaluations

A diagnosis of breast cancer must be confirmed by review of pathologic evaluation of prior biopsy and/or surgical specimen. The metastases must be documented by radiographic and/or nuclear medicine studies.

The following clinical and laboratory evaluations will occur within 30 days prior to study initiation (with the exceptions noted below). These screening evaluations must be reviewed prior to study treatment.

7.1.1 Complete history and physical examination. Include vital signs (blood pressure, pulse, temperature, and respiration), weight, and height.

7.1.2 Evaluation of Performance Status (PS) (see Appendix 1) and pain. Intensity of pain will be measured by verbal descriptors and visual analog scale (VAS) when Performance Status is measured.

7.1.3 Hematology: complete blood count (CBC) with differential, platelets, PT, and PTT.

7.1.4 Serum chemistries: glucose, electrolytes (Na+, K+, Cl-, CO_2), BUN, creatinine, total protein, albumin, bilirubin, alkaline phosphatase, LDH, SGOT, SGPT, magnesium, corrected serum calcium, phosphorus, cholesterol, and triglycerides.

7.1.5 Urinalysis.

7.1.6 Chest X-Ray (CXR).

7.1.7 Electrocardiogram (EKG).

7.1.8 CT scans of evaluable disease sites (within 60 days of study initiation).

7.1.9 Renal ultrasound (within 60 days of study initiation)

7.1.10 Bone scan (within 60 days of study initiation).

7.1.11 Pregnancy test of all patients of child bearing potential (within 7 days of study initiation). Patients will be instructed to use adequate birth control procedures throughout the study period.

7.2 Interval Evaluations

Patients will be followed in the clinic every week for the first four weeks and then every three weeks for the remainder of the study (total of 7 visits).

7.2.1 Interim history and targeted physical examination at each visit, which should include vital signs (blood pressure, pulse, temperature, and respiration), weight, and ECOG performance status (see Appendix 1).

7.2.2 Patients will be evaluated for bone pain during each visit. Intensity of pain will be measured by verbal descriptors and VAS at the same time as ECOG Performance Status is measured.

7.2.3 At each scheduled visit, each patient will be observed for possible adverse events, especially evidence of vitamin D toxicity (see Appendix 2). Any adverse event, whether observed by the investigative staff or reported by the patient, will be entered on the case report form and evaluated by the investigator as to severity and attribution. Adverse events will be documented by the criteria in Appendix 2.

7.2.4 Hematology: complete blood count (CBC), differential, and platelets at every study visit.

7.2.5 Serum chemistries: glucose, electrolytes (Na+, K+, Cl-, and CO2), BUN, creatinine, total protein, albumin, bilirubin, alkaline phosphatase, LDH, SGOT, SGPT, calcium, phosphorus, and serum lipids will be checked at every study visit. Also, 1α(OH)D5 levels and other indicators of increased blood calcium will be determined.

7.2.6 Appropriate radiographic and nuclear imaging studies will be performed at week 12 and week 28, or sooner if disease progression is suspected (see Section 11.0 and Section 17.0).

7.3 Post-treatment evaluations

7.3.1 The same evaluations as section 7.2 will be performed at one (1) month intervals for an additional four months (until week 28)

7.3.2 The same evaluations as section 7.2 will be performed every two months for an additional period of six (6) additional months.

7.3.3 Radiographic and nuclear medicine studies will be repeated at week 28 and at the end of the sixth (6) months, or sooner if disease progression is suspected.

7.4 Risks to subjects

7.4.1 Risks to subjects and measures to minimize them are listed in the table below.

Procedure	Risks	Measures to Minimize Risks
Intake of $1\alpha(OH)D_5$	Hypercalcemia, which may cause fatigue, upset stomach, constipation, nausea, bone pain, increased urination, increased thirst, weight loss, appetite loss, vomiting, low pulse rate, itching, muscle weakness, slow reflexes. At very high levels, subjects may experience confusion, mental illness, seizure or coma. Hyperphosphatemia resulting in hypocalcemia with symptoms of muscle weakness. If left untreated, could result in kidney failure	Subjects are observed for evidence of vitamin D toxicity at each scheduled visit. Blood tests will be performed every week for the first four weeks and then every three weeks during the course of treatment. At the onset of any symptoms, the subject will be tested for blood calcium and treated based on its level. Treatment usually consists of the following: 1. Administration of intravenous fluids to restore hydration. 2. Maintaining the subject's mobility and activity level consistent with the subject's presenting symptoms. 3. Administration of drugs such as diuretics or biphosphonates to lower serum calcium level.

Procedure	Risks	Measures to Minimize Risks
Venipuncture	Pain and bruising at puncture site; rarely fainting	Application of pressure at puncture site and elevation of extremity following venipuncture. Subject will be seated or lying down during blood draw.
Maintenance of data linked to an identifiable individual	Breach of confidentiality	Study participants will not be identified by name on any study documents. They will be identified by initials and a patient identification number. Investigators will keep a separate log of patients' codes, names and addresses.

8.0 TREATMENT MODIFICATION AND DISCONTINUATION ACCORDING TO LEVEL OF TOXICITY

The current NCI Common Terminology Criteria for Adverse Events table (Appendix 2, available online at http://ctep.cancer.gov/reporting/ctc.html), also applicable to vitamin D-induced hypercalcemia, will be used to grade the severity of adverse experience and to achieve consistency in response to drug/treatment toxicities. Toxicity will be graded on a 1-4 grading scale. If a toxicity is experienced, the treatment level or dose will be modified (if applicable) as outlined below according the grade toxicity observed.

8.1 Treatment modification and general management of toxicities

8.1.1 For any Grade 1 toxicity, there will be no dose modification.

8.1.2 If a Grade 2 toxicity develops, the investigator should continue the treatment with careful monitoring.

8.1.3 In general, if a Grade 3 non-hematologic or Grade 4 hematologic toxicity is obtained, treatment will be withheld until the Grade reaches 1 or less. Repeat tests to confirm values within 72 hours will be required.

8.1.4 After recovering from Grade 3 non-hematologic or Grade 4 hematologic toxicity, test medication will be restarted at a dose approximately one-half of the dose that caused the toxicity. If the original toxicity reoccurs at the lower dose level, treatment will be discontinued and the patient will be removed from study.

8.1.5 The development of Grade 3 non-hematologic or Grade 4 hematologic toxicity in two (2) out of six (6) patients at any given dose level will result in the termination of all dosing at that level and no additional higher dose cohorts will be entered into the study.

This dose level is defined as a dose limiting toxicity with the maximum tolerated dose (MTD) being one dose level below (see Section 11.1 for description of MTD).

8.2 Symptomatic therapy for toxicity

Along with reduction of the dose level, any required symptomatic therapy for hypercalcemia or other toxicity may be administered if deemed necessary by the investigators. All medications or other treatments administered will be recorded in the appropriate Case Report Form section.

9.0 CRITERIA FOR DISCONTINUATION OF STUDY PATIENTS

9.1 Criteria for treatment discontinuation

The investigator will encourage study subjects to remain in the study through completion. However, should the subject decide to withdraw, all efforts will be made to complete and report the observations as thoroughly as possible, including a complete final evaluation at the time of the subject's withdrawal with an explanation of why the subject is withdrawing from the study.

Participation in this study can be discontinued for any of the following reasons listed below:

9.1.1 Progressive disease despite an adequate trial with study medication.

9.1.2 A major, unexpected, or life-threatening event.

9.1.3 Generalized impairment or mental incompetence which would render the patient unable to understand his/her participation in the study.

9.1.4 If, in the investigator's medical judgement, further participation would be injurious to the subject's health or well-being.

9.1.5 Patient request or noncompliance.

An explanation will be recorded for any patient who has been taken off treatment, and the appropriate section of the Case Report Form will be completed.

9.2 Criteria for study modification and/or discontinuation

9.2.1 Study Modification

If preliminary or interim analysis indicates that modifications should be made in the experimental design, dosages, patient selection, etc., these changes will be made in the form of an amendment after

consultation with the sponsoring agency (Department of Defense [DOD]) and the UIC Cancer Center statistician. Changes listed in the Amendment will not be initiated until approved by the Institutional Review Committees.

9.2.2 Study Discontinuation

If the principal investigators should discover conditions arising during the study which indicate the study be terminated, an appropriate schedule for termination will be instituted and appropriate authorities notified.

10.0 ADVERSE EXPERIENCES

Both serious adverse events and serious and unexpected adverse events will be reported to the Institutional Review Board (IRB) and the Food and Drug Administration (FDA) in accordance with existing Federal Regulations. All serious adverse events and serious and unexpected adverse events will be reported immediately to:

Manley A. Paulos, Ph.D.
Vice President, Operations
Komodo Clinical Trials Management, Inc.
520 Brookview Ct. Suite 202
Auburn Hills, Michigan 48326
Voice: (248) 335-6650
Fax: (248) 335-6296
Manleypaulos@komodo-inc.com

Komodo staff will verify classification of the event, code events, and provide all written and fax/telephone notifications of events to regulatory authorities.

The event will also be immediately reported by telephone to the USAMRMC Deputy Chief of Staff for Regulatory Compliance and Quality (301-619-2165) (non-duty hours call 301-619-2165 **and** send information by facsimile to 301-619-7803). A written report will follow the initial telephone call within 3 working days. Address the written report to the U.S. Army Medical Research and Materiel Command, ATTN: MCMR-RCQ, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

11.0 EVALUATION OF RESPONSE/ENDPOINTS

11.1 Toxicity

The criteria for grading toxicity (Grade 1 through Grade 4) are found in the Toxicity Tables (see Appendix 2).

The development of a Grade 3 non-hematologic or Grade 4 hematologic toxicity in two (2) out of six (6) patients at any given dose level is defined

as a dose limiting toxicity (DLT). The maximum tolerated dose (MTD) is defined as that dosage level immediately below the level at which the DLT was observed.

11.2   Clinical response

11.2.1 Evaluation criteria

Bone Scans

Care is needed in the interpretation of a bone scan report of increased radionuclide uptake if the patient is clinically stable or improving (flare phenomenon).

Tumor Measurements

All tumor measurements must be recorded in centimeters and should consist of the longest diameter and the perpendicular diameter at the widest portion of the tumor.

Patients will be considered evaluable for toxicity if they receive one or more doses of study medication.

11.2.2 Criteria for response and definitions

Complete Response (CR) (all of the following):

-       Complete disappearance of all tumor masses. Osteolytic bone lesions must demonstrate recalcification.

-       Normalization of all laboratory parameters related to the patient's disease or to toxicity of the therapy.

-       No new lesions may appear.

-       Resolution of all symptoms related to cancer.

Partial Response (PR) (any of the following criteria):

-       A >50% decrease in the sum of the products of the diameters of any measurable lesions.

-       Recalcification of ≥ 1 osteolytic lesion.

-       A reduction by >50% in the number of areas of increased uptake on bone scan.

and ALL of the following:

-       No simultaneous increase in the size of any evaluable lesion or appearance of any new lesions.

- No deterioration in weight (10%), symptoms, or performance status (more than one score level), which is not explained by drug toxicity.

- Response duration will be measured from the time of initial documentation of response.

Stable Disease (SD) (all of the following):

- There may be no appearance of new lesions. No measurable lesion may enlarge by $\geq 25\%$.

- No elevation of serum tumor markers to >50% over baseline.

- Osteolytic lesions, if present, must not worsen.

- Osteoblastic lesions, if present, must remain stable on bone scan.

- The patient must have no significant deterioration in performance status (greater than 1 score level), weight (>10%), or symptoms.

Progressive Disease (PD) (any of the following):

- Unequivocal increase of >25% in size of any measured lesion.

- Appearance of new malignant lesions.

- Significant deterioration in weight (>10%), performance status (>1 score level), or symptoms.

Recurrence/Relapse:

- The reappearance of old lesions in patients who have achieved complete response, or, for patients with partial response, an increase of 25% or more in the sum of the products of the diameters of all measured lesions.

11.2.3 Response duration will be measured from the time of initial documentation of response.

12.0 DOCUMENTATION, RECORD KEEPING, CASE REPORT FORMS

The investigator will maintain adequate records so that the conduct of the study can be fully documented and monitored.

Copies of protocols, case report forms (CRFs), patient medical records, test result originals, and all documents relevant to the conduct of the study will be kept on file by the investigator for five years after all investigational use of

product is discontinued and the FDA is so notified or until five years after a Product Licensing Application (PLA/ELA) is approved. Study documents will not be destroyed. For FDA and sponsor inspections, it will be necessary to have access to complete study patient records, provided that patient confidentiality is maintained.

The investigator will obtain a separate release of medical information form to be signed by the study patient in order to facilitate access to the patient's medical records should the patient be hospitalized at an institution with which the study investigator is not associated.

A record will be kept of all patients who have been screened for the study and subsequently deemed ineligible. The reason for ineligibility must be recorded.

13.0 DATA COLLECTION AND STUDY MONITORING

13.1 Data collection

Case report forms (CRFs) will be used for each patient entered into the study. Study participants will NOT be identified by name on any study documents. Patients will be identified by a patient identification number (PIN). Investigators will keep a patient code list accessible.

14.0 BIOSTATISTICAL CONSIDERATIONS

The end-points of the phase I/II study are response rate and determination of the maximum tolerated dose (MTD), based on the occurrence of Grade 3 non-hematologic or Grade 4 hematologic toxicity in 2 of 6 patients at any given dose level. Should the MTD be reached before the accrual of 42 patients, the study will continue to accrue patients at one dose below the dose at which 2 of 6 patients experienced Grade 3 non-hematologic or Grade 4 hematologic toxicity.

Since the endpoints of this study are toxicity and finding both the MTD and an appropriate dose for 1-αhydroxyvitamin D$_5$, any withdrawal of a patient from any of the group will require that another patient be provided to replace that patient.

15.0 ETHICAL CONSIDERATIONS

The investigator will ensure that the study is conducted in full conformance with the FDA and the U.S. Department of Health and Human Services Office for Human Research Protections (OHRP) standards for human research.

15.1 Informed consent

All study participants must sign an informed consent form. The investigators will inform all subjects as to the nature, aims, duration, potential hazards, and procedures to be performed during the study and that his or her medical records may be reviewed by the independent monitor, UIC IRB, DOD, and/or the FDA. The investigators must also explain that the patients are completely free to refuse to enter the study or

to withdraw from it at any time. The protocol will be discussed in detail with all potentially eligible patients. All revisions of the protocol must be reflected in the consent form and reviewed by the IRB.

A translator is on staff and available to translate consent forms. This person is fluent in Spanish (the most widely used second language) and several other languages. For lesser used languages (Farsi, Cantonese, etc.), the university keeps a list of staff members fluent in these languages, and a translation can be arranged into most languages.

15.2 Patient confidentiality

All reports and patient samples will be identified only by a coded number to maintain patient confidentiality. All records will be kept confidential to the extent permitted by law. The investigators will keep a separate log of patients' codes, names, and addresses. Documents identifying the patient by name (informed consent) will be kept in strict confidence.

16.0 CRITERIA AND PROCEDURES, FOR PROTOCOL MODIFICATION AND STUDY TERMINATION

Modifications that may affect the safety of the study patient, or that may alter the scope of the investigation, the scientific quality of the study, the study design, dosages, duration of therapy, patient assessments (added evaluation that poses potential risk or inconvenience to the patient), number of patients, and patient eligibility criteria, may be made only after appropriate consultation with the UIC IRB and DOD. If the consensus is to revise the current protocol, a formal List of Changes will accompany the amended protocol, and these will be submitted to the IRB, the DOD, and any other committee as indicated. The changes to the protocol will be submitted to the FDA only in the Annual Report, which is provided within 60 days of the IND filing anniversary.

The investigators reserve the right to terminate the study at any time. If this becomes necessary, appropriate procedures for continuing the long-term follow-up requested by the regulatory agencies will be arranged after review and approval by both parties.

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17.0 SCHEDULE OF TESTS AND PROCEDURES*

Tests and procedures	Pre-study	End Wk 1	End Wk 2	End Wk 3	End Wk 4	End Wk 7	End Wk 10	End Wk 12	End Wk 16	End Wk 20	End Wk 24	End Wk 28	Q 2 months for six months or until death
History	X												
Physical Exam	X	X	X	X	X	X	X	X	X	X	X	X	X
Weight	X	X	X	X	X	X	X	X	X	X	X	X	X
Height	X												
Performance status	X	X	X	X	X	X	X	X	X	X	X	X	X
Evaluation of pain or other symptoms	X	X	X	X	X	X	X	X	X	X	X	X	X
Evaluation of toxicity		X	X	X	X	X	X	X	X	X	X	X	X
CBC, diff, PLT	X	X	X	X	X	X	X	X	X	X	X	X	X
Chemistry panel	X	X	X	X	X	X	X	X	X	X	X	X	X
Corrected Serum calcium[1]	X	X	X	X	X	X	X	X	X	X	X	X	X
Serum phosphate[2]	X	X	X	X	X	X	X	X	X	X	X	X	X
Urinalysis	X												
Chest X-Ray	X												
EKG	X												
Bone scan	X							X			X	**
Ultrasound of kidney	X							X			X	**
CT scan	X							X			X	**
Pregnancy test[3]	X												
D5 levels		X	X	X	X	X	X	X	X	X	X	X	

* Weekly tests will be run for 28 weeks, though treatment will only last 12 weeks (plus six months of follow-up tests every two months). All tests will be run at the end of the week indicated plus or minus two (2) days.
** Radiographic and nuclear medicine studies as indicated above or sooner if disease progression is suspected.
[1,2,3] will be done within one week of initiation of the study.

APPENDIX 1

ECOG CRITERIA FOR ESTIMATION OF PERFORMANCE STATUS

CRITERIA FOR ESTIMATION OF PERFORMANCE STATUS

Grade Scale

0   Fully Active, able to carry on all pre-disease performance without restriction.

1   Ambulatory, capable of light or sedentary work. Restricted in physically strenuous activity.

2   Ambulatory, capable of all self-care, but not of work activities; up and about more than 50% of waking hours.

3   Capable of only limited self-care; confined to bed or chair more than 50% of waking hours.

4   Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.

APPENDIX 2

TOXICITY TABLE

THE NCI COMMON TERMINOLOGY CRITERIA FOR ADVERSE EVENTS (CTCAE)

Available online at http://ctep.cancer.gov/reporting/ctc.html

APPENDIX 3

STRUCTURE OF 1α-HYDROXY-24, ETHYL CHOLECALCIFEROLE (1α(OH)D$_5$)
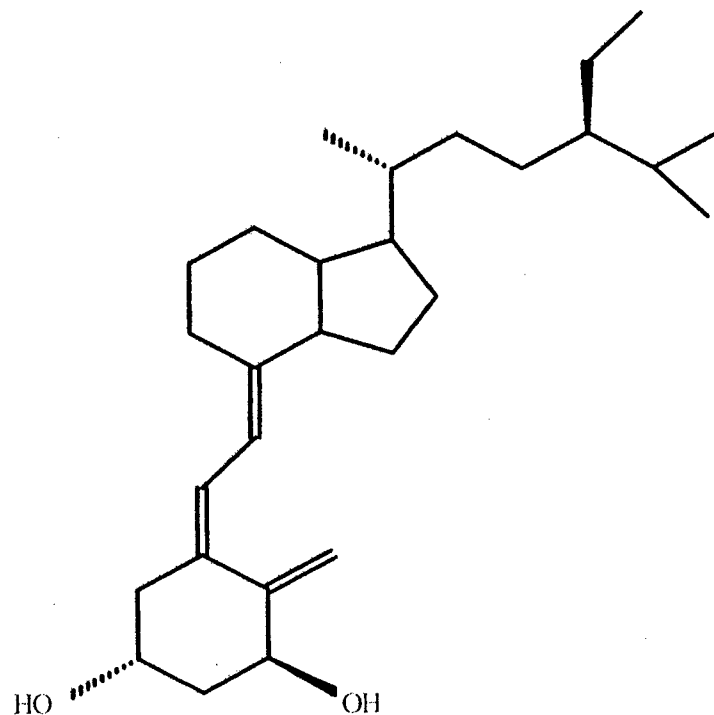
Chemical structure and high-pressure liquid chromatography (HPLC) profile of 1αhydroxyvitamin D_5 [1α(OH)D_5]. The agent was dissolved in acetonitrile (200 μg/mL), and l0-μL aliquots were injected on a Suplex PKB-1OO HPLC column. The retention time for 1α(OH)D_5 was about 34 minutes.

SAMPLE INFORMATION			

Sample Name:	D5 std	Acquired By:	System
Sample Type:	Unknown	Date Acquired:	2/13/03 10:50:41 AM
Vial:	12	Acq. Method Set:	Vitamin D
Injection #:	1	Date Processed:	2/21/03 1:00:06 PM
Injection Volume:	100.00 ul	Processing Method:	Default
Run Time:	45.0 Minutes	Channel Name:	474 Ch1, 486
Sample Set Name:	D5 day 7	Proc. Chnl. Descr.:	
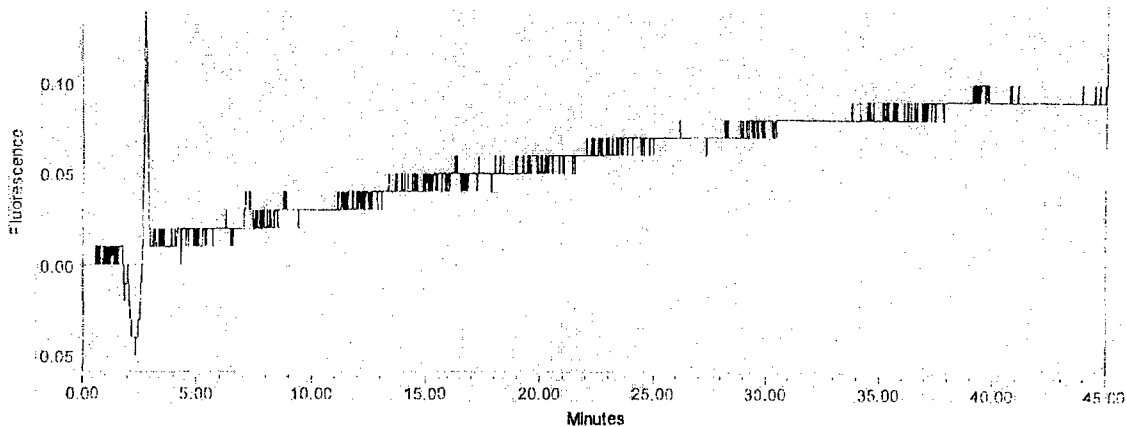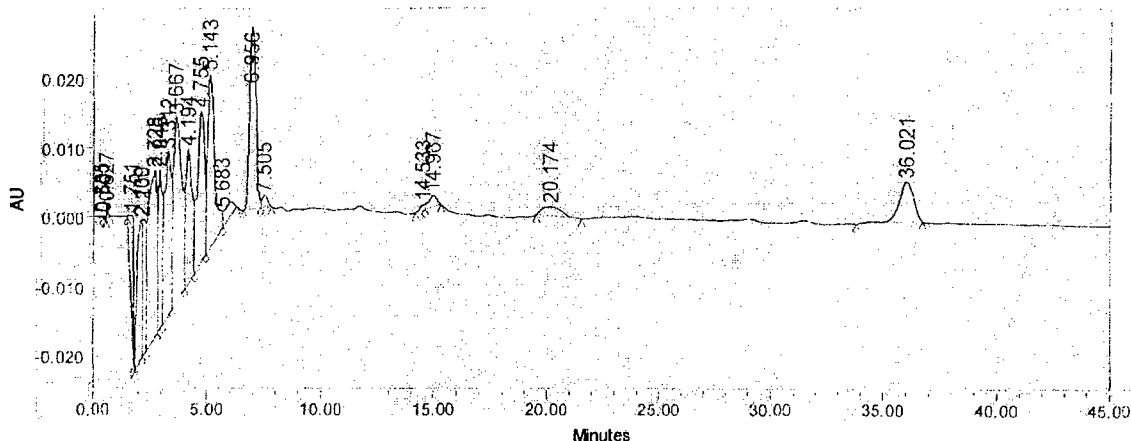
	Peak Name	RT	Area	% Area	Height
1		0.385	5321	0.10	348

Report Method: Result Set Report   Printed 1:00:09 PM 2/21/03

001--082

Page: 1 of 6

M

Reported by User: System

Project Name: Vit_D1

	Peak Name	RT	Area	% Area	Height
2		0.501	2246	0.04	489
3		0.627	8595	0.17	1883
4		1.751	168922	3.26	16641
5		2.139	321667	6.21	20220
6		2.200	192888	3.72	19810
7		2.728	638507	12.32	24279
8		2.946	268769	5.19	23157
9		3.312	515961	9.96	23900
10		3.667	687136	13.26	26898
11		4.194	377511	7.29	19414
12		4.755	482298	9.31	21973
13		5.143	514082	9.92	25195
14		5.683	58130	1.12	2415
15		6.956	524665	10.13	26296
16		7.505	30655	0.59	1734
17		14.533	13187	0.25	860
18		14.967	42976	0.83	1613
19	MDA	18.000			
20	MDA	18.000			
21		20.174	75701	1.46	1306
22		36.021	252242	4.87	5786

1αhydroxyvitamin D_5 Phase I/II Trial Protocol

(Version #10 (06/30/04) – Page 30 of 33

APPENDIX 4

Protocol for Compounding Final Dosage Form

PROCEDURE FOR VITAMIN D5 STORAGE AND PREPARATION OF CAPSULES TO BE USED IN CLINIC UNDER IND # 56,509

1. Pure Vitamin D5 (drug substance) is stored at -75 °C (Revco freezer model #ULT 390-5-A14 Revco Elite) with continuous graphic recording of temperature. Graphic recording charts are changed regularly and maintained on file.

2. On the day capsules are to be prepared, Vitamin D5 is removed from the freezer for formulation in cornstarch. D5 is dissolved in ethanol for dilution in cornstarch, according to the procedure outlined by Dr. Raju Mehta.

   a. Weigh 1 mg Vitamin D5 (using Acculab V-1 Electronic Balance)
   b. Dissolve D5 in 1 mL 100% ethanol, USP
   c. Add 1 mL of corn oil, USP
   d. Add 0.5 mL Tenox® for a final concentration of 1 mg/2.5 mL

3. Preparation of 5 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 0.25 mL D5 solution (0.1 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 5 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

4. Preparation of 10 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 0.5 mL D5 solution (0.2 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 10 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

5. Preparation of 15 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 0.75 mL D5 solution (0.3 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 15 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

6. Preparation of 20 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 1.0 mL D5 solution (0.4 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 20 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

7. Preparation of 25 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 1.25 mL D5 solution (0.5 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 25 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

8. Preparation of 30 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 1.5 mL D5 solution (0.6 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 30 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

9. Preparation of 35 μg dosage:

   a. Weigh 3 gm of cornstarch, NF
   b. Using a syringe, measure 1.75 mL D5 solution (0.7 mg)
   c. Using a mortar and pestle, hand mix the D5 solution with the cornstarch for 2 minutes for a final concentration (w/w) of 35 μg D5/150 mg cornstarch
   d. Weigh 150 mg of D5/cornstarch mixture to fill each of 52 capsules (size 0; Apothecary Products, Inc.)

Prepared capsules will be immediately placed in vials (7 per vial), labeled with the CTM label, and transported to the clinical sites. Ten (1 a) capsules per batch will be placed in a vial with the CTM label, and marked "RETAINS", and stored at room temperature under lock and key in the pharmacy.

Appendix 2: Publications (3)

Efficacy and Mechanism of Action of 1α-hydroxy-24-ethyl-Cholecalciferol (1α[OH]D5) in Breast Cancer Prevention and Therapy

Erum A. Hussain · Rajeshwari R. Mehta · Rahul Ray · Tapas K. Das Gupta · Rajendra G. Mehta

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Abstract

It is now well established that the active metabolite of vitamin D_3, $1\alpha,25(OH)_2D_3$, regulates cell growth and differentiation in various in vitro cancer models. However, its clinical use is precluded due to its hypercalcemic activity in vivo. Hence, several less calcemic vitamin D analogs have been synthesized and evaluated for their chemopreventive and therapeutic efficacy in experimental carcinogenesis models. A novel analog of vitamin D_3, 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5), has currently been under investigation in our laboratory for its application in breast cancer prevention and therapy. 1α(OH)D5 had been shown to inhibit development of estrogen- and progesterone-dependent ductal lesions as well as steroid hormone-independent alveolar lesions in a mammary gland organ culture (MMOC) model. Moreover, the inhibitory effect was more significant if 1α(OH)D5 was present during the promotional phase of the lesion development. The growth inhibitory effect of 1α(OH)D5 has also been manifested in several breast cancer cell lines, including BT-474 and MCF-7. Breast cancer cell lines that responded to 1α(OH)D5 treatment were vitamin D receptor positive (VDR+). Vitamin D receptor-negative (VDR−) cell lines, such as MDA-MB-231 and MDA-MB-435, did not show growth inhibition upon incubation with 1α(OH)D5. This suggests the requirement of VDR in 1α(OH)D5-mediated growth effects. Interestingly, breast cancer cells that were VDR+ as well as estrogen receptor positive (ER+) showed cell cycle arrest and apoptosis, while VDR+ but ER− cells (UISO-BCA-4 breast cancer cells) showed enhanced expression of various differentiation markers with 1α(OH)D5 treatment. Transcription and expression of estrogen-inducible genes, progesterone receptor (PR) and trefoil factor 1 (pS2), were significantly down-regulated in ER+ BT-474 cells with 1α(OH)D5 treatment. This implies a differential effect of 1α(OH)D5 on ER+ vs. ER− cells. Additionally, comparison between the effects of 1α(OH)D5 on normal vs. transformed cells indicated that 1α(OH)D5 does not suppress cell prolifera-

tion of normal epithelial cells but selectively targets growth of transformed cells. We extended our experiments to determine in vivo effects of 1α(OH)D5 using an MNU-induced mammary carcinogenesis model in female Sprague-Dawley rats. Results showed that 1α(OH)D5 (25–50 μg/kg diet) decreased the incidence and multiplicity of mammary tumors in these rats. In addition, it increased the latency period of early precancerous lesions. Similar studies, with DMBA as a carcinogen in younger rats, showed that 1α(OH)D5 supplementation was effective in reducing onset of carcinogenesis in rats and the effect was largely reflected during the promotional phase of carcinogenesis. Recently, a preclinical toxicity profile for 1α(OH)D5 was completed in rats and dogs that provides estimation of the maximum tolerated dose in mammals. Based on our findings, we will shortly be initiating a 1α(OH)D5 phase I clinical trial for breast cancer patients.

Introduction

Breast cancer is generally characterized by transformation of normal to atypical hyperplastic epithelium, with subsequent risk of progression to intraductal carcinoma and in some cases invasion into stroma (Mallon et al. 2000). Breast cancer is the second leading cause of cancer-related deaths among women in the US, with about 180,000 new cases and 46,000 deaths annually (Edwards et al. 2002). Although the overall incidence of breast cancer has not been reduced in the last decade, the breast cancer-related mortality has been decreasing with approximately 3.4% annual decrease from 1995 through 1998 in the US (Howe et al. 2001; Peto et al. 2000). This decrease in mortality is probably a result of availability of greater screening efficiency and better chemopreventive and therapeutic strategies. Despite increased survival rates, breast cancer results in considerable morbidity and patient care costs. Chemoprevention is an important aspect of curbing the progression or recurrence of the disease. The chemopreventive agents usually include natural or synthetic compounds that can either prevent transformation or inhibit proliferation of transformed cells by inducing apoptosis, growth arrest or differentiation of initiated and transformed cells (Rosenbaum-Smith and Osborne 2000). Several classes of compounds have been under investigation in this regard. These include selective estrogen receptor modulators, retinoids, deltanoids (vitamin D derivatives), phytoestrogens, flavonoids, and aromatase inhibitors, among others (Kelloff et al. 1996). ·

On a global basis, breast cancer incidence is fivefold higher among middle-aged women in the Western countries than in women from Asian countries. Various diet and lifestyle as well as genetic factors have been implicated in the high occurrence of breast cancer in the Western world. Some epidemiological studies have shown association of lower sunlight exposure to higher breast, colon, and prostate cancer mortality rates in the US and other Western countries (Freedman et al. 2002; Polek and Weigel 2002; Garland et al. 1990; Gorham et al. 1990). This is consistent with reports of an association of breast

cancer mortality with lower serum vitamin D_3 levels (John et al. 1999; Christakos 1994). Lower serum vitamin D_3 levels could be due to lower sunlight exposure as well as lower dietary intake.

The biologically active metabolite of vitamin D, $1\alpha,25(OH)_2D_3$ or calcitriol, is a steroid hormone that was identified in the early 1920s as an antirachitic substance (Carpenter and Zhao 1999). Later it was established that vitamin D_3 is synthesized in the skin from 7-dehydrocholesterol by the action of ultraviolet radiation. Vitamin D_3 is activated subsequently in liver and kidney by the hydroxylation reactions at C25 and 1α positions to yield $1\alpha,25(OH)_2D_3$. Calcitriol has been known to exert calciotrophic effects, mainly through increasing calcium uptake in the intestine for regulation of bone health. Aside from its role in calcium homeostasis, vitamin D_3 is involved in regulation of various cellular processes. Vitamin D_3 binds to nuclear vitamin D receptor (VDR) and undergoes conformational changes, which allow VDR to function as a transcription factor (Jones et al. 1998; Haussler 1986). Earlier, VDR was found to be present in abundance in intestine, bone, liver, and kidney cells. Aside from the classic target organs, VDR has now been isolated from a variety of tissues, including normal mammary epithelium as well as breast tumors (Friedrich et al. 1998; Buras et al. 1994; Eisman et al. 1980).

In order for VDR to function, it needs to interact with vitamin D response elements (VDRE) and bind to DNA to initiate or repress transcription (Pike 1991). VDR must form a dimer to stabilize VDRE transactivation (Jones et al. 1998). Most common partners for VDR heterodimerization are nuclear accessory factor (NAF) and retinoid X receptor (RXR) (Rachez and Freedman 2000). VDR transactivation of VDRE results in regulation of a wide variety of genes, some of which are involved in cell growth and proliferation. Vitamin D_3 also exerts some nongenomic rapid responses possibly through a putative membrane receptor (Falkenstein et al. 2000).

The presence of VDR in the normal mammary epithelial cells suggests a role of calcitriol in the regulation of mammary gland function. The levels of VDR in mammary tissue increase during pregnancy and lactation and decrease as the glands regress back to normal size (Zinser et al. 2002; Narvaez et al. 2001). VDR knockout mice have been shown to have larger mammary glands than normal mice; it has also been shown that the glands would not regress back to prepregnancy size at the termination of lactation (Zinser et al. 2002). This suggests that vitamin D mediated signaling may be very important for maintaining the normal cycling of the mammary gland. Various case studies indicate that a high percentage (60%–80%) of breast cancer epithelia contain VDR (Friedrich et al. 1998) and that there is a positive correlation between VDR polymorphisms and increased risk of breast cancer (Bretherton-Watt et al. 2001; Lundin et al. 1999). These reports further signify vitamin D_3-mediated signaling to be of importance in regulating healthy mammary gland. In cell culture models, vitamin D_3 has been demonstrated as an inducer of growth arrest and differentiation in various cancer cell lines, including breast cancer cells (Hisatake et al. 2001; Welsh et al. 1998; James et al. 1997). Taken together, these results warrant potential use of vitamin D_3 in cancer preven-

tion and therapy. However, due to its hypercalcemic activity, vitamin D_3 can not be administered at doses that would be effective for chemoprevention or therapy. Adverse effects of vitamin D_3 at cancer-preventive doses are hypercalcemia, soft tissue calcification, weight loss, and possibly death (Roder and Stair 1999; Vieth, 1999).

Since the early 1980s, there has been a search for a vitamin D_3 analog that would selectively modulate VDR to produce growth-regulating effects without interfering with the calcium metabolism. Several analogs have been synthesized and tested for this purpose; but only a few have shown promising results in cell culture and animal models. Vitamin D_3 analogs currently being evaluated for breast cancer prevention include seocalcitol (EB-1089), calcipotriol (KH-1060), Maxacalcitol (OCT), RO-24-5531, and 1α(OH)D5 (Mehta and Mehta 2002; Guyton et al. 2001). In this review, we summarize the results from experiments conducted in our laboratory that elucidate the potential role of 1α-hydroxy-24-ethyl-cholecalciferol (1α[OH]D5) in breast cancer prevention or therapy.

Synthesis and Characterization of Vitamin D Analog, 1α(OH)D5

As mentioned earlier, vitamin D_3 can be obtained from food as well as synthesized in the skin through the action of sunlight. Vitamin D_3 belongs to the family of 9,10-secosteroids which differ only in side-chain structure (Napoli et al. 1979). Other forms of D-compounds include D2, D4, D5, and D6. In the late 1970s, major interest in the synthesis of these compounds was to evaluate them for use in management of renal osteodystrophy and osteoporosis. In this regard the calcemic activity of D series of compounds was compared and D5 was found to be the least calcemic of all (Napoli et al. 1979), a property that would later prove useful in its possible application for cancer prevention. The D5 form is also known as irridated 7-dehydrositosterol. The hydroxylated form of D5 (1α[OH]D5) was synthesized as described previously (Mehta et al. 1997a).

Briefly, β-sitosterol acetate was converted to 7-dehydro-β-sitosterol acetate by allelic bromination and dehydrobromination. Lithium aluminum hydride and tetrahydrofurane were used to reduce 7-dehydro-β-sitosterol to 7-dehydro-3β-sitosterol. The reaction mix was sequentially subjected to photolysis and thermolysis to yield 24-ethyl-cholecalciferol (D5). D5 was hydroxylated by Paaren-DeLuca hydroxylation sequence to produce 1α(OH)D5. The product was crystallized and characterized by ^{1}H nuclear magnetic resonance at 400 Hz and mass spectroscopy. The purity was assessed by high-pressure liquid chromatography. The following properties were observed: melting point, 150–152°C; UV λ-max, 265 nm; molar extinction coefficient (ε), 18000; molecular weight, 428.7. The major structural differences between biologically active vitamin D_3 and 1α(OH)D5 are the lack of hydroxylation at the C-25 position and the presence of an ethyl group at the C-24 position in the 1α(OH)D5 molecule (Fig. 1).

Structural Difference from
1α(OH)₂D₃

• 1α(OH)D5 has an extra
  ethyl group at C 24

• 1α(OH)D5 does not posses
  hydroxyl group at C 25

Calcemic Activity
$$D_3 = D_2 > D_4 > D_6 > D_5$$

1 α -hydroxy-24-ethyl-
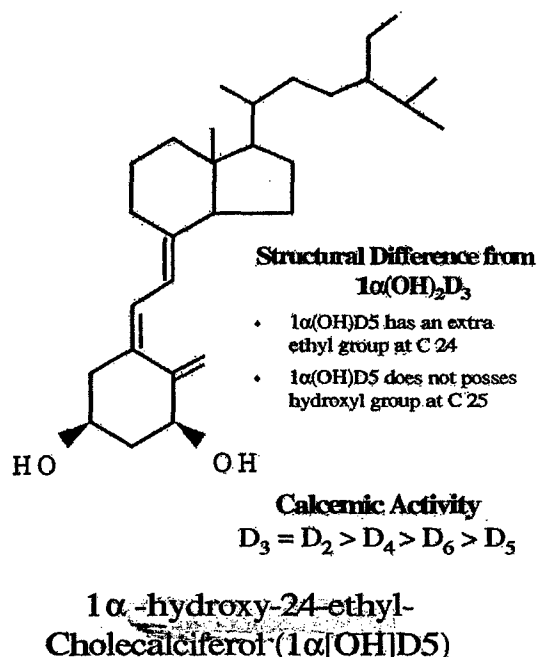Cholecalciferol (1α[OH]D5)

Fig. 1 Structure of 1α(OH)D5 and its Ca⁺⁺ mobilizing activity in mammals in relation to other primary vitamin D series compounds

Calcemic Activity of 1α(OH)D5

Earlier studies in DeLuca's lab had shown that among the known vitamin D series of compounds (vitamin D_2–D_6), D5 is the least calcemic of all (Napoli et al. 1979). D5 was found to be 80-fold less active than vitamin D_3 in the intestine and about 100- to 200-fold less active in bone in mobilizing the Ca^{++} stores (Napoli et al. 1979). The calcemic activity of the hydroxylated form was not known. Therefore, we measured calcemic activity as well as body weight change in animal models to determine the maximum tolerable dose and toxicity of 1α(OH)D5. In the first experiment, 3-week-old Sprague-Dawley male rats were fed a vitamin D_3-free diet containing 0.47 g calcium and 0.3 g phosphorus/100 g diet (Mehta et al. 1997a). These rats were kept under yellow light to create a vitamin D_3-deficiency state. After the rats were fed a vitamin D_3-deficient diet for 3 weeks, their plasma calcium levels were measured and rats with calcium levels under 6.0 mg/dl were considered vitamin D_3 deficient. Vitamin D_3-deficient rats were administered 1α(OH)D5 intragastrically for 14 days and the plasma calcium levels were measured. The control group showed a plasma calcium concentration of 5.4+0.3 mg/dl, while the rats receiving 1α(OH)D5 at a dose of 0.042 μg/kg per day had plasma calcium concentration of 6.0+0.63 mg/dl, which was not significantly different from the control rats (Mehta et al. 1997a). On the other hand, vitamin D_3 increased

Table 1 Calcemic activity of 1α(OH)D5 in Sprague-Dawley rats

* Significantly different from control (*p* <0.05)

plasma calcium concentration 50% over that of the control group (Table 1). During these experiments, the 1α(OH)D5 group did not differ in total body weight from control group. No other signs of toxicity were observed in 1α(OH)D5-fed rats compared to controls.

In a separate experiment, female Sprague-Dawley rats were fed a diet supplemented with 1α(OH)D5 to determine its calcemic activity in vitamin D_3-sufficient rats. Food was provided ad libitum. There was no body weight change at 50 μg 1α(OH)D5/kg diet in vitamin D_3-sufficient rats, while a dose of 12.8 μg 1α,25(OH)$_2$D3/kg diet was sufficient to bring about significant weight loss in the animals (Table 1). Maximum tolerated dose was determined to be 50 μg/kg diet, based on the weight and calcemic activity of 1α(OH)D5 in these rats (Mehta et al. 2000a). In addition to these experiments, we also conducted toxicity studies under the GLP using rats and dogs. For rats, the dose at which signs of toxicity first appeared was 10 μg/kg body weight (equivalent to 100 μg 1α(OH)D5/kg diet for a 150-g rat), which is twice the amount needed to bring about effective chemoprevention. However, the dogs had much lower tolerance for 1α(OH)D5 compared to rats. Based on these results, we are now conducting further studies to determine the appropriate and safe dose of 1α(OH)D5 for use in clinical settings.

Since vitamin D_3 exerts most of its effects through binding to VDR, we evaluated the ability of 1α(OH)D5 to bind to VDR. The binding affinity of 1α(OH)D5 to VDR was determined using competitive binding assays (unpublished data). Results showed that the binding affinity of 1α(OH)D5, in competition with radioactive 1α,25(OH)$_2$D$_3$ to purified VDR ligand-binding domain is 1000-fold less than 1α,25(OH)$_2$D$_3$ (Fig. 2). The IC$_{50}$ for 1α(OH)D5 was 100 pM, while for 1α,25(OH)$_2$D$_3$, it was 0.08 pM. The lower binding affinity may explain the decreased calcemic activity of 1α(OH)D5. However, due to its lower calcemic activity, 1α(OH)D5 can be administered at much higher doses than 1α,25(OH)$_2$D$_3$. This quality can allow use of 1α(OH)D5 for prevention in the general population as well as high-risk groups. It is also important to note that the in vivo VDR affinity to its ligand is tissue specific (Napoli et al. 1979), which could not be manifested in our experiments that were conducted using
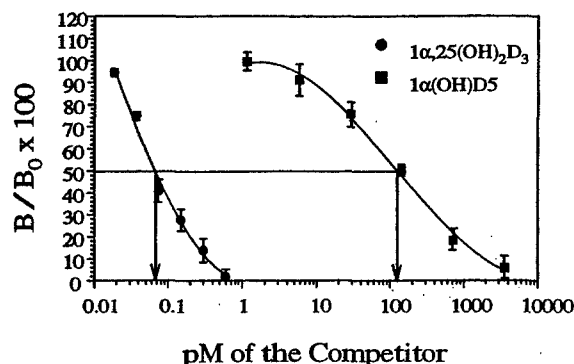
Fig. 2 Binding affinity of 1α(OH)D5 to VDR in comparison with 1α,25(OH)$_2$D$_3$

purified VDR. We have not yet critically evaluated metabolism and pharmacokinetics of 1α(OH)D5 in target organs.

Anticarcinogenic Effects of 1α(OH)D5 in In Vitro Models

The effectiveness of a variety of chemopreventive agents has been evaluated by organ culture of the mouse mammary gland (MMOC). The mammary glands from balb/c mice are harvested and cultured in presence of appropriate hormones (Mehta et al. 1997b). These glands are subjected to short stimulation with a carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA), which results in formation of precancerous preneoplastic lesions. When implanted in syngeneic hosts, the epithelial cells from these lesions give rise to adenocarcinomas. Effective chemopreventive agents would inhibit the development of these preneoplastic lesions. The chemopreventive activity of a compound in MMOC correlates very well with the activity in in vivo carcinogenesis models (Mehta et al. 1997b). Using a DMBA-induced MMOC model, Mehta et al. (1997a) showed that 1α(OH)D5 possesses chemopreventive activity. Fifteen mammary glands (per group) from balb/c mice were incubated with appropriate hormones and were exposed to the carcinogen DMBA (2 μg/ml of culture media) on day 3 of a 24-day culture. The group of glands incubated with 1α(OH)D5 showed significant reduction of lesion formation compared to the control group (Fig. 3). Percent inhibition of lesion formation in each treatment group was calculated by comparing the incidences of lesions between the control and the treated group. A dose–response curve showed that 100% inhibition was achieved at 10 μM 1α(OH)D5 concentration, but the optimal dose seems to be 1 μM, as it shows significant (75%) inhibition without any signs of cytotoxicity. Vitamin D$_3$, on the other hand, caused dilation of ducts and disintegration of alveolar structures as signs of toxicity at 1 μM concentration. Based on the MMOC model, 1 μM 1α(OH)D5 seems to be equivalent in potency to 0.1 μM 1α,25(OH)$_2$D$_3$.
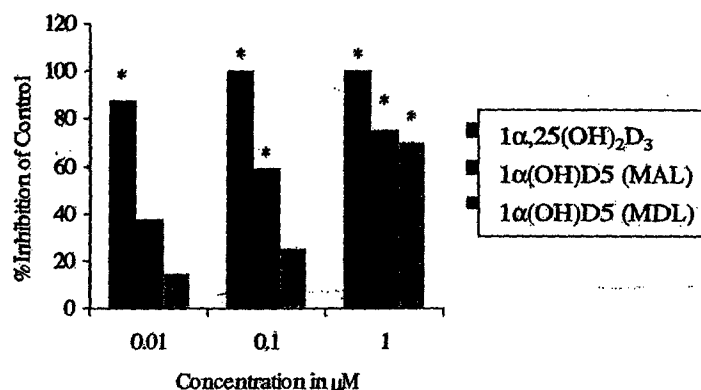
Fig. 3 Chemopreventive efficacy of 1α(OH)D5 in inhibiting mammary alveolar (MAL) and ductal (MDL) lesions in mouse mammary gland organ culture in comparison to 1α,25(OH)₂D₃

In order to establish the stage specificity for the effectiveness of 1α(OH)D5 in a DMBA-induced MMOC model, 1α(OH)D5 was added either prior to or subsequent to carcinogen treatment. The initiation-only group received 1α(OH)D5 for the first 4 days of culture, whereas the promotion-only group received the treatment after withdrawal of carcinogen (days 4–10). Results indicated that 1α(OH)D5 is more effective when present during the promotional stages of lesion formation (Mehta et al. 2000a). In addition to inhibition of lesion formation, 1α(OH)D5 was effective in inducing VDR and TGFβ1 expression in mammary epithelial cells of MMOC. VDR and TGF-β1 expression was measured using immunohistochemistry. Briefly, paraffin-embedded sections were rehydrated, fixed, permeabilized, and incubated with primary antibody. The primary antibody binding was detected using biotinylated link and peroxidase-conjugated streptavidin, which was then visualized by 3-amino-9-ethyl-carbazole as chromogen. The mammary epithelial cells, which stained negative for VDR, failed to show TGF-β1 induction upon 1α(OH)D5 treatment. This implies the involvement of VDR in 1α(OH)D5-mediated effects. The extent of induction of VDR and TGF-β1 upon treatment with 1.0 μM 1α(OH)D5 was similar to that observed with 0.1 μM vitamin D₃ (Mehta et al. 1997a). Despite the 1000-fold lower affinity of 1α(OH)D5 for VDR in comparison to 1α,25(OH)₂D3, its chemopreventive activity is equivalent to 1α,25(OH)₂D₃ at only a 100-fold higher concentration. Therefore, it seems likely that the antiproliferative effects of 1α(OH)D5 may not be dependent solely upon its in vitro interactions with VDR.

Since the MMOC experiments involved the whole organ, the actions of 1α(OH)D5 on breast epithelia itself were not clearly established. Hence, we tested the growth effects of 1α(OH)D5 on various breast cancer cell lines of epithelial origin. All the cell lines tested were purchased from ATCC (Manassas, VA, USA), except UISO-BCA-4 cells. This cell line was established in our laboratory from metastatic pleural fluid obtained from a 56-year-old woman with a confirmed diagnosis of breast carcinoma (Mehta et al. 1992). The

Table 2 Growth response of various breast cancer cell lines to 1α(OH)D5 treatment

[a] Percent growth inhibition at 1 μM 1α(OH)D5 for 72 h, adjusted for control

growth effects of 1α(OH)D5 were assessed on BT-474, MCF-7, ZR-75–1, T-47D, UISO-BCA-4, MDA-MB-231 and MDA-MB-435 cell lines using multiple measures: cell counter, MTT absorbance assay (Twentyman and Luscombe 1987), and cell cycle analysis with propidium iodide staining and flow cytometry (Vindelov et al. 1983). The overall effects of 1α(OH)D5 on the growth of different cell lines are summarized in Table 2. All the cell lines that were positive for VDR showed significant growth inhibition ($p<0.05$) after 72 h of incubation with 1α(OH)D5. BT-474, and MCF-7 (VDR+ ER+ PR+) cells showed the greatest growth inhibition and G-1 cell cycle arrest upon 1α(OH)D5 treatment. Similarly, UISO-BCA-4 (VDR+ ER– PR–) cells exhibited growth inhibition in response to 1α(OH)D5 treatment. On the other hand, VDR– MDA-MB-231 and MDA-MB-435 cells did not show any growth inhibition at 1 μM 1α(OH)D5 treatment (Mehta et al. 2002). The dose–response curve for 1α(OH)D5 effect in BT-474 cells was similar to that observed in the MMOC experiments.

Chemopreventive Efficacy of 1α(OH)D5 in In Vivo Carcinogenesis Models

Once we established the in vitro efficacy of 1α(OH)D5, the effects of 1α(OH)D5 were evaluated in experimental mammary carcinogenesis models. We used mammary-specific carcinogen N-methyl-N-nitrosourea (MNU) in rats to induce tumors and evaluated the efficacy of 1α(OH)D5 to prevent or delay the incidence of mammary cancers in these rats (Mehta et al. 2000a). Fifteen Sprague-Dawley female virgin rats per group (9 weeks old) were fed 1α(OH)D5-supplemented diet (25 or 50 μg/kg) for 2 weeks before the carcinogen treatment. The carcinogen MNU was given as a single intravenous injection of 50 mg acidified MNU/kg body weight at 80 days of age. The rats continued to receive the 1α(OH)D5-supplemented diet until they were killed at 190 days of age. The tumor incidence in control rats was 80%, which, compared to controls, decreased in 25- and 50-μg/kg diet group by 33% and 42%, respectively (Table 3). The tumor incidence in the low-dose group was not sig-

Table 3 Efficacy of 1α(OH)D5 in preventing carcinogenesis in animal models

ᵃ Duration in weeks
ᵇ 8 ng 1α(OH)D5 subcutaneously injected thrice weekly for 60 days
ᶜ Significantly different from control ($p<0.05$)
ᵈ Results are expressed as tumor volume (cm³)

nificantly reduced from control ($p=0.12$), whereas the high-dose group had a significantly lower tumor incidence ($p=0.03$). However, when the three groups were compared using log-rank analysis, the comparison reached statistical significance ($p=0.0495$). Tumor multiplicity was not significantly different between the control group and the 25-μg/kg diet group, but it was significantly lower in the high-dose group ($p=0.02$).

The encouraging results from MNU-carcinogenesis model prompted us to extend our in vivo experiments. Since MNU is a direct-acting carcinogen, we chose another mammary-specific carcinogen that needs to be metabolized, such as DMBA. For the DMBA carcinogenesis study, 7-week-old rats (20 per group) were given 15 mg DMBA intragastrically. 1α(OH)D5 was supplied in the diet (20–40 μg/kg diet) 2 weeks prior to carcinogen treatment. The control group showed 85% tumor incidence and the high-dose group showed 60% incidence, while the low-dose group showed a significant decrease in incidence (40%). Table 3 summarizes the results from in vivo experiments. Although the high-dose group did not show a significant decrease in tumor incidence, it had significantly lower tumor multiplicity (0.6 compared to 1.9 in the control group). Moreover, the chemopreventive efficacy of 1α(OH)D5 was more pronounced when provided at progressional stages of the disease.

In addition to assessing chemopreventive properties of 1α(OH)D5 in mammary carcinogenesis, we evaluated its efficacy as a possible chemotherapeutic agent. These experiments were carried out in xenograft models, as previously described (Mehta and Mehta 2002). Initial studies were conducted using xenograft of UISO-BCA-4 cells pretreated with 1 μM 1α(OH)D5 for 10 days, which failed to form tumors in athymic (4-week-old) mice. In other studies, UISO-BCA-4 cells were xenografted in athymic mice and either 8 ng 1α(OH)D5 per animal was injected IP thrice a week or 1α(OH)D5 was provided in the diet at

12.5 μg/kg diet for 6 weeks. All the animals in the control group formed tumors whereas only one of the treated animals showed a scab-like structure at injection site in the IP group. Forty percent of controls showed metastasis to lymph nodes but 1α(OH)D5 treatment prevented metastasis of cells transplanted in athymic mice (Mehta and Mehta 2002). In the dietary treatment group, 1α(OH)D5 inhibited growth of UISO-BCA-4 cells and the tumor volume was suppressed to nearly 50% of control. Similar results were obtained with BT-474 xenograft in athymic mice. These results suggest that 1α(OH)D5-induced cell growth inhibition and differentiation is protective against tumor growth in the xenograft model as well.

Growth Response of Normal versus Transformed Cells to 1α(OH)D5

While we established that 1α(OH)D5 has growth inhibitory action on cancer cells, the effects on normal breast epithelial cells were not known. In order to determine that, we cultured mammary glands from mouse with appropriate hormones in the absence of any carcinogens. Ten glands were treated with 1α(OH)D5 and other glands were used as controls. At the end of 6-day culture, the glands were terminated, paraffin embedded, and sectioned for pathological evaluation. Histopathological examination showed no difference in the growth and morphology of glands treated with 1α(OH)D5 from that of control glands. In view this result, we evaluated the effects of 1α(OH)D5 on MCF-12F cells, which are nontumorigenic breast epithelial cells derived from reduction mammoplasty from a 60-year-old Caucasian woman. These cells were spontaneously immortalized by long-term culture in low-Ca^{++} media. To determine their growth response, MCF-12F cells were incubated with 1α(OH)D5 for various intervals, but no growth inhibitory effect was observed at the 1-μM concentration.

To establish selectivity of 1α(OH)D5 effects on transformed or preneoplastic cells, we transformed MCF-12F cells with DMBA and MNU to study if the transformation status could affect the response to 1α(OH)D5. Transformation was performed using the protocol described elsewhere (Lazzaro et al. 1997). Briefly, passage 10 MCF-12F cells were grown to subconfluency in tissue culture dishes and incubated with DMBA (2 μg DMBA/ml culture media) for 24 h. The procedure was repeated the next day. Extensive cell death resulted. The surviving cells were allowed to grow in fresh medium and later selected out with serum starvation. The resulting cell line was designated MCF-12F$_{DMBA}$. Similarly, in another experiment, MNU was dissolved in acidified saline (pH 5.3) and added to subconfluent MCF-12F cells at a concentration of 25 μg/ml twice daily for 2 days. The surviving cells were allowed to grow and the new cell line was established after serum starvation. These cells were called MCF-12F$_{MNU}$. The growth rate and morphological characteristics were compared between these cell lines. The growth rates of transformed cells were three times higher than MCF-12F. By the fifth passage of postcarcinogen treatment, the MCF-12F$_{DMBA}$ doubling time was reduced to one-third of MCF-12F,

Table 4 Growth effects of 1 μM 1α(OH)D5 on normal and transformed MCF-12F breast epithelial cells

* Significantly different from control ($p<0.05$)

while for MCF-12F$_{MNU}$, it was reduced to one-fourth of MCF-12F. Moreover, the transformed cell lines did not exhibit the contact inhibition characteristic of the normal cells.

As mentioned earlier, MCF-12F cells showed no growth inhibitory response with 1α(OH)D5 treatment. The transformed cells, on the other hand, showed significant growth inhibition (60% for MCF-12F$_{MNU}$ and 40% for MCF-12F$_{DMBA}$), as determined by the MTT absorbance assay. Other measures of growth provided similar results (Table 4). These studies indicate that the transformed cells respond differently to 1α(OH)D5 treatment than the parent cell line.

Potential Mechanism of Action of 1α(OH)D5 in Breast Cancer Prevention and Therapy

Previously mentioned studies have implicated the involvement of VDR in 1α(OH)D5-mediated growth effects. VDR− highly metastatic cells such as MDA-MB-231 and MDA-MB-435 do not respond to 1α(OH)D5 treatment. Moreover, mammary epithelial cells which lack VDRs also fail to respond to 1α(OH)D5 and do not show induction of VDR and TGF-β1 (Mehta et al. 1997a). VDR+ breast cancer cells, such as T-47D, had been shown to increase transcription of VDR upon incubation with 1α(OH)D5 as determined by RT-PCR (Lazzaro et al. 2000). This VDR induction was not observed in the cell line BT-474, either at transcription or expression levels, upon treatment with 1α(OH)D5. A possible explanation could be the high constitutive levels of VDR present in this cell line. To ascertain VDR-mediated VDRE transactivation activity of 1α(OH)D5, we used the CAT reporter gene containing VDRE (VDRE-tk-CAT). For this purpose, CV-1 monkey renal cancer cells were used as these lack a functional VDR. After VDRE-tk-CAT transient transfection into CV-1 cells, 1α(OH)D5 could not induce the CAT activity in these cells. But when the cells were cotransfected with VDRE and VDR, there was an enhanced expression of CAT activity, suggesting the capability of 1α(OH)D5 to activate VDR-mediated signaling. The relative CAT activity in CV-1 cells that had been cotransfected with VDRE and VDR was 200,000-fold higher than control when treated with 0.1 μM 1α(OH)D5 (Lazzaro et al. 2000).
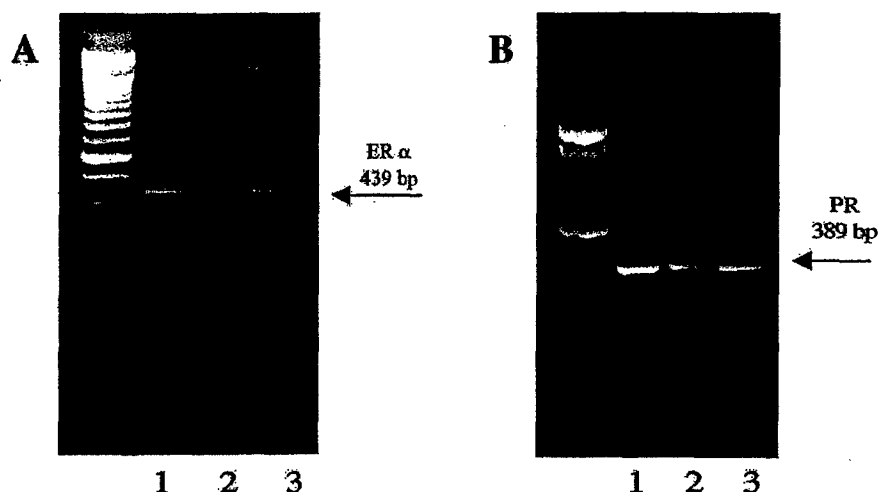
Fig. 4 Down-regulation of estrogen- (A) and progesterone- (B) receptor transcription with vitamin D_3 and its analog in BT-474 cells as determined by RT-PCR. Lane 1 control, lane 2 1α,25(OH)$_2$D$_3$, lane 3 1α(OH)D5

Breast cancer UISO-BCA-4 cells are ER− and PR−, but VDR+. These cells responded differently to 1α(OH)D5 than the ER+ cells (Mehta et al. 2003). UISO-BCA-4 cells were treated with 0.1 μM 1α(OH)D5 for 10 days. The 1α(OH)D5 treatment resulted in induction of intracytoplasmic casein granules, increased lipid droplets, ICAM-1, α2-integrin, nm23, and VDR, manifesting the differentiation markers. Use of this cell line allows us to determine estrogen-independent effects of 1α(OH)D5. While 1α(OH)D5 induced differentiation in ER− cells, it induced apoptosis in ER+ BT-474 and MCF-7 cells, as determined by acridine orange/ethidium bromide staining and TUNEL assay (Mehta et al. 2003). In both these cell lines, there is a G-1 cell cycle arrest followed by apoptosis.

Because the actions of 1α(OH)D5 differ in ER+ breast cancer cells, we examined the effects of 1α(OH)D5 on estrogen-dependent signaling in the ER+ PR+ BT-474 cells. BT-474 cells showed down-regulation of both ER and estrogen-inducible PR transcription upon treatment with 1α(OH)D5, as determined by RT-PCR (Fig. 4). This was in turn followed by down-regulation at the expression level, as estimated by immunocytochemistry (Fig. 5). These results are consistent with reports by other researchers that describe the role of vitamin D_3 in down-regulation of estrogen-inducible genes (Swami et al. 2000; Stoica et al. 1999). The vitamin D_3-VDR pathway may be a negative feedback mechanism to regulate the estrogen-induced proliferation of the mammary tissue. Some researchers have postulated an interaction of VDR-D_3 to ERE to repress the estrogen-mediated gene transcription (Welsh et al. 1998; Demirpence et al. 1994).

Fig. 5a, b Down-regulation of progesterone receptor (PR) expression with 1α(OH)D5 treatment in BT-474 cells as detected by immunocytochemical analysis. Percentage of cells stained positive for PR were 78% in control (a) and 46% in treated cells (b)
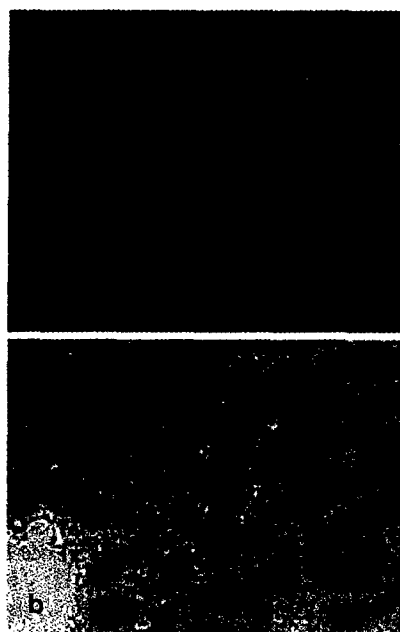
Since vitamin D_3 is known to regulate a wide variety of genes, we investigated other potential gene targets of 1α(OH)D5 in BT-474 cells. The microarray was performed using Human UniGene 1 by Incyte Genomics, Inc. (Palo Alto, CA, USA), which contained 8,000 genes along with appropriate controls. Among the major targets of 1α(OH)D5 were the estrogen-inducible genes PR, trefoil factor 1 (pS2), and trefoil factor 3 ($p<0.05$). A few selected genes that were statistically significantly altered are presented in Table 5.

As mentioned earlier, the transformed MCF-12F cells showed growth inhibition even though these cells express very low levels of steroid receptors. It is possible that other mechanisms are at work to bring about growth arrest in MCF-12F$_{DMBA}$ and MCF-12F$_{MNU}$ cells. Therefore, we used Clontech Atlas microarrays (Genomics Inc.) with 10,000 genes to identify differentially expressed genes in the transformed MCF-12F$_{MNU}$ cells as compared to the MCF-12F parent cell lines. In a second comparison, we assessed the genes differentially expressed by 1α(OH)D5 treatment in MCF-12F$_{MNU}$ cells. Interestingly, many genes that were differentially expressed in MCF-12F$_{MNU}$ cells compared to the MCF-12F cells were altered inversely in 1α(OH)D5 treated MCF-12F$_{MNU}$ cells (Table 5). Most of the genes that were affected were transcription-related and mitochondrial genes. Of interest are proteins such as vimentin, prohibitin, MAPK-7, and HSP-27, which are usually expressed at higher levels in mammary tumors (Atanaskova et al. 2002; Zajchowski et al. 2001; Storm et al. 1996). These proteins were down-regulated in 1α(OH)D5-treated cells. Differentiation-related proteins such as integrins and cadherins were up-regulated by 1α(OH)D5 in both BT-474 and MCF-12F$_{MNU}$ cell systems.

Table 5 Microarray analysis to determine effects of 1 μM 1α(OH)D5 and MNU-induced transformation on selected genes

Prohibitin might be a potentially important vitamin D_3-regulated protein, which was found to be more highly expressed in the transformed MCF-12F cells than the parent cell line (data not shown). Some studies have shown high prohibitin levels in tumor tissue and cancer cell lines (Jupe et al. 1996; Asamoto and Cohen, 1994). However, the role of this mitochondrial protein is controversial. Wang and co-workers (1999) have shown its involvement in regulation of the cell cycle, whereas others have shown that the levels do not represent the cell cycle-related functions but rather are indicative of mitochondrial stress (Coates et al. 2001). It is possible that the mitochondrial stress may be indicative of the higher proliferative rates of the transformed cells. Another protein of interest was thioredoxin, which was up-regulated in MCF-$12F_{MNU}$ cells and down-regulated by 1α(OH)D5 treatment. Thioredoxin is a redox protein with growth factor activity that modulates the activity of several proteins important for cell growth. Some researchers have observed increased thioredoxin transcription and expression in primary human tumors (Matsutani et al. 2001; Berggren et al. 1996). Administration of inhibitors of thioredoxin system has been shown to have antitumor activity in vivo (Kirkpatrick et al. 1999). Moreover, Gallegos and co-workers (1996) reported that transfec-
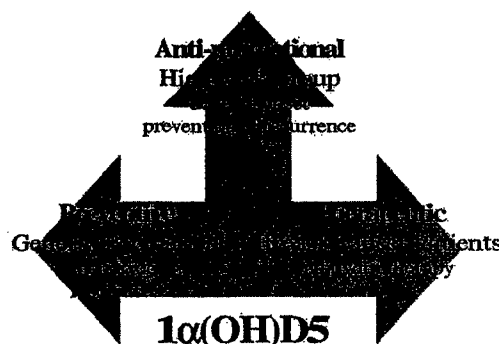
Fig. 6 Potential application of 1α(OH)D5 in breast cancer prevention and therapy

tion of dominant-negative mutant thioredoxin resulted in reversal of transformed phenotype of human breast cancer cells. Therefore, it appears that the mechanism of action of 1α(OH)D5 involves multiple genes and pathways, some of which have not yet been thoroughly investigated. Further studies are needed to elucidate the mechanism of action of 1α(OH)D5 in normal and cancer breast cells.

Conclusions

Results presented in this report on effects of 1α(OH)D5 are suggestive of its promise in chemoprevention. 1α(OH)D5 has consistently been shown to be effective in inhibiting growth of cancer cells as well as preneoplastic lesions in mammary glands in vitro. The in vitro effects are manifested in vivo as well. In the animal carcinogenesis models, 1α(OH)D5 had reduced the incidence of tumors as well as tumor multiplicity, and increased the latency period. Yet there were no changes in total body weight and no apparent signs of toxicity at efficacious doses. More recently, we completed preclinical toxicity studies in rats and dogs under good laboratory practices and regulations, providing an estimation of maximum tolerable dose. The concentration of 1α(OH)D5 required to achieve optimal cell regulatory effects is 100 times higher than the concentration of vitamin D_3. However, there is no hypercalcemia observed at this dose of 1α(OH)D5 to warrant concern. The mechanism of action of 1α(OH)D5 seems to involve VDR as well as cross-talk with the estrogen signaling pathway. It has been shown to inhibit estrogen-induced proliferation. Because of these properties, 1α(OH)D5 might prove suitable in a variety of applications. Furthermore, the differential gene expression profile clearly suggested that the effects of 1α(OH)D5 involve multiple pathways and genes, some of which have not yet been critically studied.

A scheme of possible applications of 1α(OH)D5 is presented in Fig. 6. From a prevention point of view, 1α(OH)D5 might be used in populations that are at high risk or to prevent or delay recurrence of breast tumors in breast cancer

patients. It might also be used in conjunction with other treatments for cancer therapy. Further studies are underway in our laboratory to determine if indeed 1α(OH)D5 would become available for clinical use in the future.

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Chemoprevention of mammary carcinogenesis by 1α-hydroxyvitamin D₅, a synthetic analog of Vitamin D

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Abstract

Numerous analogs of Vitamin D have been synthesized in recent years with the hope of generating a compound that retains the anticarcinogenic activity of Vitamin D without causing any toxicity. We synthesized such an analog, 1α-hydroxy-24-ethylcholecalciferol [1α-hydroxyvitamin D₅ or 1α(OH)D₅], and showed that it was tolerated by rats and mice at a much higher dose than 1α,25 dihydroxy cholecalciferol [1α,25(OH)₂D₃]. This property makes it a prime candidate for chemoprevention studies. In the mouse mammary gland organ culture (MMOC), 1α(OH)D₅ inhibited carcinogen-induced development of both mammary alveolar and ductal lesions. In vivo carcinogenesis study showed statistically significant reduction of tumor incidence and multiplicity in N-methyl-N-nitrosourea (MNU)-treated rats that were fed 25–50 μg 1α(OH)D₅/kg diet. There were no adverse effects on plasma calcium concentrations. In order to determine if the effect of 1α(OH)D₅ would be selective in suppressing proliferation of transformed cells, its effects on cell growth and proliferation were compared between BT474 (cancer) and MCF12F (non-tumorigenic) human breast epithelial cells. Results showed that 1α(OH)D₅ induced apoptosis and cell cycle G1 phase arrest in BT474 breast cancer cells without having any effects on proliferation of the MCF12F cells. In addition, in MMOC it had no growth inhibitory effects on normal epithelial cell proliferation in the absence of carcinogen. Similarly, non-tumorigenic human breast epithelial cells in explant culture did not respond to 1α(OH)D₅, whereas treatment with 1α(OH)D₅ induced cell death in the explants of cancer tissue. These results collectively indicate that 1α(OH)D₅ selectively induced apoptosis only in transformed cells but not in normal breast epithelial cells. Interestingly, the growth inhibitory effects of 1α(OH)D₅ were observed in Vitamin D receptor positive (VDR⁺) breast cancer cells, but not in highly metastatic VDR⁻ breast cancer cells, such as MDA-MB-435 and MDA-MB-231, suggesting that 1α(OH)D₅ action may be mediated, in part, by VDR.

1. Introduction

Conceptually, chemoprevention of cancer can be defined as an intervention in the carcinogenic process by either a naturally derived or a synthetic compound. An agent that blocks, arrests, or reverses the progression of cancer can be termed a chemopreventive agent [1,2]. In practice, this can best be achieved by the dietary administration of chemical agents, which can enhance the physiological processes that protect the organism against the development of malignancy. Current understanding of progression of a normal

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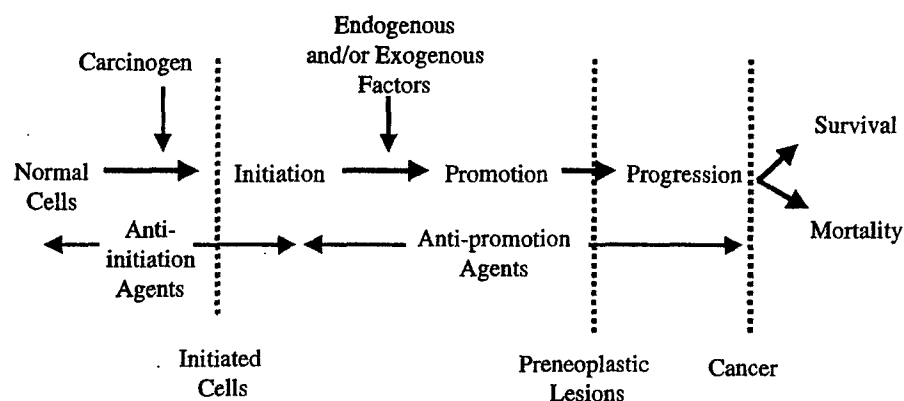*E-mail address:* raju@uic.edu (R.G. Mehta).

Fig. 1. Schematic diagram to show stages in mammary carcinogenesis and potential points of intervention by chemopreventive agents.

cell to a transformed cancer cell is summarized in Fig. 1. Under experimental conditions, a normal cell could be transformed to an initiated cell in response to carcinogenic or mutagenic stimuli. Although the initiated cells have the potential to develop into malignant cancer, they may or may not form a tumor depending upon exposure to exogenous and/or endogenous factors. In the absence of growth arrest stimuli, the initiated cell can advance to a preneoplastic stage leading progressively to malignancy. The chemopreventive agents that suppress the early events in transformation, such as preventing the mutagenic action of chemicals or other factors, are referred to as anti-initiation agents. On the other hand, chemicals that prevent further progression of initiated cells into transformed ones are termed anti-promotional agents [3,4]. Numerous classes of chemopreventive agents have been reported in the literature, including retinoids, deltanoids, cyclooxygenase inhibitors, inhibitors of polyamine and prostaglandin biosynthesis, lignans, calcium channel blockers, anti oxidants, etc. [5–7]. In this report, we have summarized the chemopreventive properties of a newly evaluated Vitamin D analog, 1-α-hydroxy-24-ethyl-cholecalciferol [1α(OH)D$_5$].

It has been well established that the active metabolite of Vitamin D, 1α,25-dihydroxyvitamin D$_3$, [1,25(OH)$_2$D$_3$] is a steroid hormone and it exhibits potent cell-differentiating properties in leukemia cells as well as other cancer cells of epithelial origin [8,9]. The antiproliferative and differentiation-inducing effects of 1,25(OH)$_2$D$_3$ could be of clinical signifi-

cance in prevention or treatment of cancer of several target organs [10]. However, one major limitation in its clinical application is the fact that the efficacious concentrations of 1α,25(OH)$_2$D$_3$ are cytotoxic [11]. The effective growth inhibitory concentration of 1α,25(OH)$_2$D$_3$ induces dangerously high levels of serum calcium resulting in loss of body weight and soft tissue calcification, which could be lethal [12]. This has resulted in generation of several non-toxic but antiproliferative synthetic analogs of the Vitamin D molecule for the prevention and treatment of cancer. Some of these analogs have been successfully evaluated for their ability to suppress cancer cell growth in culture as well as in vivo models [13].

Typically, the structure of Vitamin D is divided into four parts (Fig. 2): ring A, open ring B, ring CD, and the side chain. Modifications can be made at all four sites, but the alteration of the ring CD is not common due to its rigid structure. Most alterations have been made at the open side chain. Nearly 800 analogs of Vitamin D have been synthesized so far, and about 300 of them have been evaluated in in vitro and in vivo experimental models [14,15]. Historically, a comparison of the toxicological profile of the Vitamin D series of compounds, including D$_2$–D$_6$, had suggested that D$_5$ was the least toxic of the D series of compounds [16]. In order to generate an effective but non-calcemic and non-toxic Vitamin D analog, we synthesized 1α(OH)D$_5$ [17]. The structure of 1α(OH)D$_5$ is shown in Fig. 2.

Vitamin D hormone mediates its action by both genomic and non-genomic pathways. The genomic

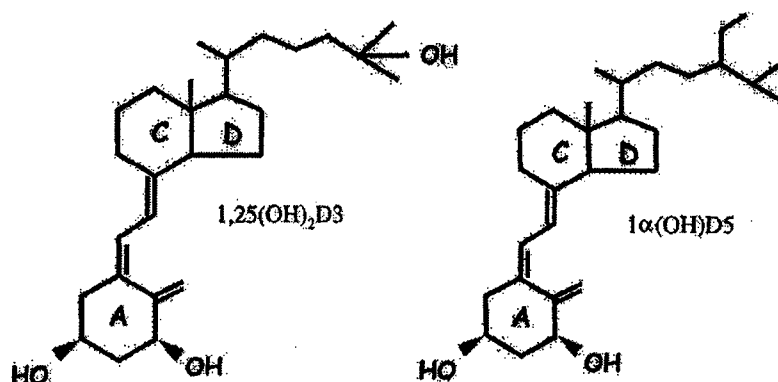Fig. 2. Structural representation of 1,25(OH)$_2$D$_3$ and its analog 1α(OH)D$_5$.

pathway involves its association with high-affinity specific Vitamin D receptor (VDR) that belongs to the steroid receptor superfamily of ligand-activated transcription factors [18–20]. This is consistent with the well-known mode of action of the steroid hormones. The VDR has been identified in a variety of tissues such as breast, prostate, liver, fibroblasts, colon, and lungs [21], in addition to the previously known target organs that included intestine, kidney, and bone.

The VDR mRNA is about 4.6 kb, which translates to a 50-kd protein in humans. The VDR content ranges from 400 to 27,000 copies per cell, yielding 10–100 fmoles/mg of total protein. In order for VDR to function, it needs to bind specific DNA sequences and interact with Vitamin D response elements (VDRE) [22]. The natural metabolite 1α,25(OH)$_2$D$_3$ transactivates VDRE in VDR$^+$ cells but fails to show interaction in VDR$^-$ cells. Hence, Vitamin D analogs that are able to transactivate VDR–VDRE are mainly mediating their action via genomic pathways. Non-genomic Vitamin D actions have been studied mostly in relation to calcium and phosphorus metabolism, and to a lesser extent with respect to chemoprevention. The rapid responses involve a putative membrane receptor of Vitamin D that signals to modulate calcium channel activity in a cell. This may lead to exocytosis of calcium-bearing vesicles from lysosomes. The non-genomic pathway for Vitamin D action has been extensively reviewed elsewhere [23,24]. For this article, we have listed the chemopreventive properties and possible mode of action of 1α(OH)D$_5$.

2. Materials and methods

2.1. Cell lines

We purchased from the American Type Culture Collection (ATCC), Bethesda, MD and maintained in our laboratory according to the ATCC recommendations the following cell lines: (1) the non-tumorigenic, estrogen receptor-negative (ER$^-$), progesterone receptor-negative (PgR$^-$), and low VDR breast epithelial cell line MCF12F; (2) ER$^+$, PgR$^+$, and VDR$^+$ breast cancer cell lines BT474 and MCF7; and (3) ER$^-$, PR$^-$, and VDR$^-$ breast cancer cell lines MDA-MB-231 and MDA-MB-435.

2.2. Mouse mammary gland organ culture (MMOC)

The detailed procedures for culturing mammary glands from Balb/c mice have been previously reported in the literature [17,25] and outlined in Fig. 3. Briefly, thoracic pairs of mammary glands from Balb/c mice are maintained in serum-free Waymouth's MB752/1 medium under 95% O$_2$ and 5% CO$_2$ at 37 °C. The glands respond to growth-promoting hormones insulin, prolactin, aldosterone, and hydrocortisone and differentiate into distinct alveolar structures. Exposure of glands to 7,12-dimethylbenz(a)anthracene (DMBA) for 24 h on day 3 of culture results in the development of precancerous mammary alveolar lesions (MAL). If the growth-promoting medium contains estrogen and progesterone instead of aldosterone and hydrocortisone, the
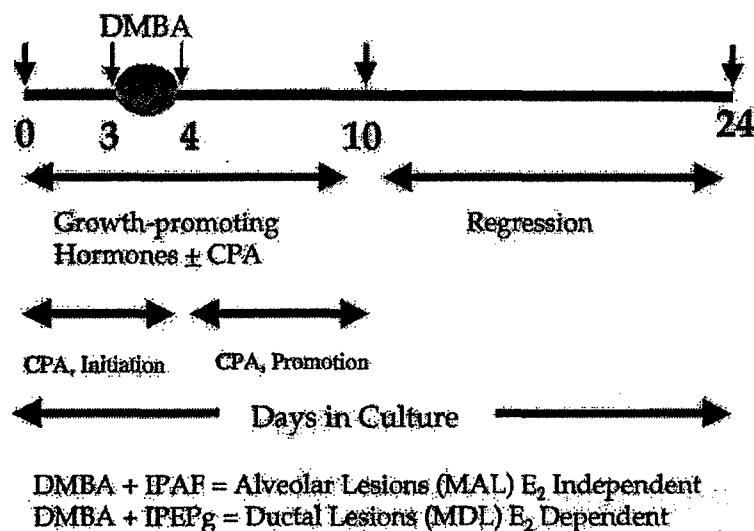
Fig. 3. Experimental design for chemoprevention in mouse mammary gland organ culture (MMOC). DMBA: 7,12-dimethylbenz(a)anthracene, CPA: chemopreventive agent, IPAF: insulin + prolactin + aldosterone + hydrocortisone, IPEPg: insulin + prolactin + estradiol + progesterone, MAL: mammary alveolar lesions, MDL: mammary ductal lesions.

glands develop mammary ductal lesions (MDL) with DMBA treatment [26]. We performed a dose response study to compare the effects of $1\alpha(OH)D_5$ on MAL and MDL. Mammary lesions developed in the absence of $1\alpha(OH)D_5$ served as controls. Additionally, we determined the effects of $1\alpha(OH)D_5$ on normal mammary glands, where the glands were incubated with growth-promoting hormones and 1 µM $1\alpha(OH)D_5$ for 6 days without DMBA treatment. The glands from these MMOC experiments were fixed, stained, and analyzed for morphological characteristics and cell growth and compared with the appropriate controls.

2.3. Cell cycle analysis by flow cytometry

To determine cell cycle, we used flow cytometric analysis as described by Vindeløv et al. [27]. Breast epithelial non-tumorigenic and cancer cells were detached by trypsinization and were harvested. The cells were washed twice with PBS and pelleted. The pellet was resuspended and fixed in 85% ice-cold ethanol. After fixing, the cells were centrifuged and resuspended in citrate buffer and then incubated with NP-40, trypsin, and spermine for 15 min. This was followed by incubation with trypsin inhibitor and RNAase A. The cells were then stained with 0.04% propidium iodide solution. Approximately

10,000 cells were analyzed for DNA content using a Beckman-Coulter EPICS Elite ESP flow cytometer. Multicycle analysis software was used to determine the percentage of cells in various stages of cell cycle. Each experiment was repeated twice and student's t-test was used to assess differences.

2.4. Apoptosis

Programmed cell death was evaluated using acridine orange staining. Briefly, a 50 µl suspension of breast epithelial cells was stained with 2 µl of acridine orange/ethidium bromide solution (100 µg/ml acridine orange and 100 µg/ml ethidium bromide in PBS). Cells were layered on a glass slide and examined under a fluorescent microscope with a 40× objective lens using a flourescein filter. Approximately 100 cells were counted on each slide to assess the proportion of cells undergoing apoptosis.

2.5. Mammary carcinogenesis

The procedure for induction of mammary adenocarcinomas by N-methyl-N-nitrosourea (MNU) in Sprague–Dawley female rats has been described in detail previously [28] and is illustrated in Fig. 4. Briefly, 100-day-old female Sprague–Dawley rats
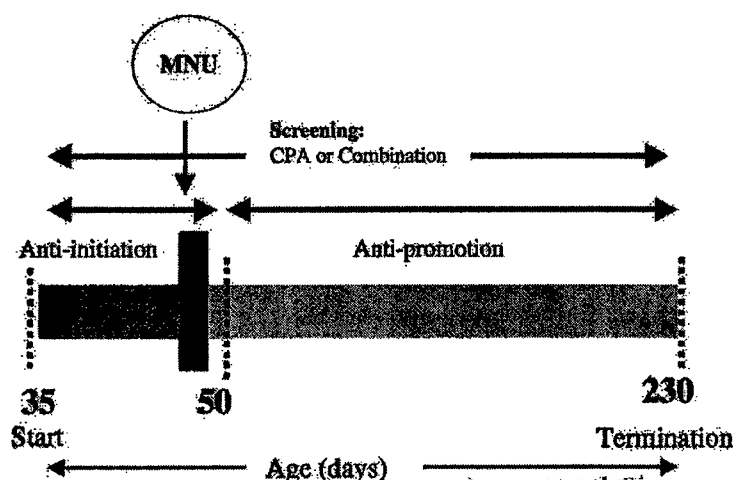
Fig. 4. Schematic diagram to show in vivo model of chemoprevention in N-methyl-N-nitrosourea (MNU)-induced mammary carcinogenesis in Sprague–Dawley rats. CPA: chemopreventive agent.

were injected subcutaneously with 50 mg/kg MNU prepared in acidified saline. Animals received either placebo or $1\alpha(OH)D_5$ supplemented as 25 or 50 µg/kg diet. Animals were sacrificed after 230 days of treatment. Mammary tumors were identified by palpation as well as necroscopy. Results were reported as effects of $1\alpha(OH)D_5$ on the incidence, multiplicity, and latency of tumor development, and data were subjected to appropriate statistical analyses.

2.6. Effects of $1\alpha(OH)D_5$ on normal and malignant breast tissue

Breast tissues were obtained from women undergoing mastectomy or lumpectomy. Explants were maintained in MEME medium, containing 5% stripped fetal bovine serum. The effects of 1 µM $1\alpha(OH)D_5$ were determined on these tissues by evaluating cell morphology, apoptosis, and expression of Ki 67. The effects of $1\alpha(OH)D_5$ on cell morphology and Ki 67 were compared between the normal and adjacent cancer tissue from the same patient.

2.7. Statistical analysis

Statistical analyses were performed using Graph-Pad Instat® 3.0 software. All MMOC as well as MNU-induced carcinogenesis data were evaluated using χ^2 analysis. Cell viability, apoptosis, and cell cycle results were assessed using two-tailed student's t-test with type I error set at 0.05. Serum calcium and phosphorus data were tested with student's t-test as well. All in vitro experiments were performed in duplicates and repeated twice.

3. Results and discussion

3.1. Synthesis and toxicity of $1\alpha(OH)D_5$

Nearly 300 analogs of $1,25(OH)_2D_3$ have been evaluated in various experimental systems in the hope of generating analogs that are more efficacious with reduced toxicity. Among the analogs evaluated, only a few have shown potent chemopreventive and therapeutic activity. These analogs include EB1089 [29], KH1060 [30], RO24-5531 [31], and 22-Oxacalcitriol [32], which are relatively nontoxic at effective concentrations in experimental models. The hexafluoro analog of $1,25(OH)_2D_3$, RO24-5531, has no calcemic activity, while other analogs do express dose-related calcemia [33,34]. Since it had been reported previously that Vitamin D_5 is the least toxic series of Vitamin D compounds, we synthesized $1\alpha(OH)D_5$ with the intention of testing its chemopreventive potential. The chemical synthesis of $1\alpha(OH)D_5$ has been previously reported from our laboratory [17].

Since calcemic activity is an obstacle to the development of effective Vitamin D analogs suitable for clinical use, we determined serum calcium and phosphorous concentrations after treating Vitamin D-deficient rats with $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$. As reported earlier, male Sprague–Dawley rats (8–10 per group) were fed Vitamin D-deficient diet for 3 weeks, and baseline serum calcium levels were determined. Rats showing <6 mg/dl serum calcium were given $1\alpha(OH)D_5$ for 14 days. Subsequently, serum calcium concentrations were measured. Results showed that $1,25(OH)_2D_3$ significantly ($P < 0.001$) increased serum calcium concentration at a daily dose of 0.042 μg/kg diet, whereas there was no elevation in serum calcium levels among $1\alpha(OH)D_5$-treated animals [17].

A similar experiment was carried out using Vitamin D-sufficient regular diet. Female Sprague–Dawley rats were treated with various concentrations of $1,25(OH)_2D_3$ (0.8–12.8 μg/kg diet) and $1\alpha(OH)D_5$ (6.4–50 μg/kg diet) for 2 months. Calcium concentration was increased by $1,25(OH)_2D_3$ treatment, while no serum calcium elevation was observed in $1\alpha(OH)D_5$-treated (25 μg/kg diet) animals (Table 1). There was no effect on the final body weight at any dose of $1\alpha(OH)D_5$ used in this study. These results indicate that $1\alpha(OH)D_5$ is considerably less toxic compared to the natural hormone.

More recently, we completed an extensive preclinical toxicity study in both sexes of rats and dogs under good laboratory practice (GLP). Results showed that dogs are relatively more sensitive to the higher dose of $1\alpha(OH)D_5$ than are rats. We concluded from those studies that $1\alpha(OH)D_5$ is calcemic in dogs at concentrations higher than 10 μg/kg diet. The non-calcemic analog RO24-5531 shows toxicity in rats without having an effect on serum calcium concentrations. On the other hand, $1\alpha(OH)D_5$ can be tolerated at a higher concentration without other toxicity outcomes.

Chemoprevention of mammary carcinogenesis by $1\alpha(OH)D_5$: The chemopreventive properties of $1\alpha(OH)D_5$ have been evaluated in two experimental systems in our laboratory. These include MMOC and MNU-induced mammary carcinogenesis in Sprague–Dawley rats. Mouse mammary glands respond to DMBA and develop preneoplastic mammary alveolar as well as ductal lesions in organ culture. As shown in Fig. 3, the efficacy of a potential chemopreventive agent can be assessed in this assay. If the agent is present and effective prior to carcinogen treatment, its effects are considered as anti-initiation, whereas, if it is effective subsequent to carcinogen, then its effect are anti-promotional. Both types of effects can be determined using the MMOC model.

We showed previously that $1\alpha(OH)D_5$ inhibits the development of mammary lesions in a dose-responsive manner [17]. However, it requires 10-fold higher concentration than the effective concentration of $1,25(OH)_2D_3$. The most effective dose of $1,25(OH)_2-D_3$ in suppressing >60% incidence of MAL is 10^{-7} M, while $1\alpha(OH)D_5$ is equally effective at 10^{-6} M without showing cytotoxicity. We also evaluated $1\alpha(OH)D_5$ effects in the MDL model [25]. The results are summarized in Fig. 5. We found $1\alpha(OH)D_5$ to be equally effective against alveolar and ductal lesions.

Since most of the effects of Vitamin D are mediated through VDR, we determined VDR induction by $1\alpha(OH)D_5$ in MMOC as well as in breast cancer cell lines [17]. There was a significant increase in the expression of VDR in the epithelial cells of MMOC as determined by immunocytochemistry. Additionally, $1\alpha(OH)D_5$ also upregulated the expression of TGFβ in the epithelial cells of MMOC [15].

Based on these results, it was reasonable to expect chemopreventive activity of $1\alpha(OH)D_5$ in an in vivo model. Prior to conducting in vivo carcinogenesis studies, a dose tolerance study was conducted in Sprague–Dawley rats. Animals were provided with increasing concentrations of $1\alpha(OH)D_5$, ranging from

Table 1
Effects of $1\alpha(OH)D_5$ treatment on serum calcium and phosphorous levels in Sprague–Dawley rats ($n = 10$)

Agent	Dose (μg/kg)	Serum Ca (mg/dl)	Serum P (mg/dl)[a]	BW (% gain)
None		6.3	3.6	100
$1,25(OH)_2D_3$	0.8	7.0	6.4	101
	3.2	7.1	8.0	104
	12.8	7.5*	8.9	70*
$1\alpha(OH)D_5$	6.4	6.3	7.2	99
	12.5	6.2	7.2	97
	25.0	6.5	7.1	98
	50.0	ND	ND	113

* Significantly different from control ($P < 0.05$).
[a] Significance not determined.
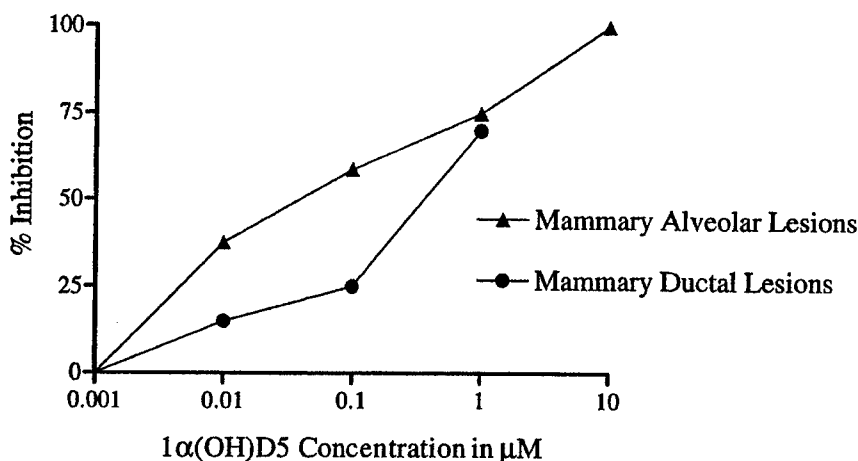
Fig. 5. Effect of 1α(OH)D5 on mouse mammary organ culture (MMOC). The glands were incubated with 1 μM 1α(OH)D5 for 10 days. The glands were fixed and evaluated for inhibition of preneoplastic lesions in relation to control. Fifteen glands were used per group. A difference in inhibition of greater than 60% was considered significant ($P < 0.05$, χ^2). Data shows significant inhibition of preneoplastic MAL and MDL with 1α(OH)D5 treatment.

1 to 100 μg/kg diet for 6 weeks. The animals did not show any adverse effects at any concentration of 1α(OH)D5, while the natural hormone was toxic at 3.5 μg/kg diet.

For the MNU-induced mammary carcinogenesis studies, animals were fed 1α(OH)D5 at 25 and 50 μg/kg diet for 3 months. The experimental diet was given to the animals 1 week prior to the carcinogen treatment and continued until the end of the study. Results are shown in Table 2. The results indicated a dose-dependent suppression of tumor incidence by 1α(OH)D5. This was accompanied by a reduction in tumor multiplicity and an increase in tumor latency [28]. These results are comparable with those of EB1089, R024-5531, and KH1060. The in vivo results as well as the results from MMOC clearly suggest a potential for 1α(OH)D5 to be developed as a chemopreventive and therapeutic agent.

Table 2
Chemoprevention of MNU-induced mammary carcinogenesis by 1α(OH)D5 in rats

Treatment	Dose (μg/kg)	n	Incidence (%)	Multiplicity	Final BW (g)
Control	0	15	80	1.6	228
1α(OH)D5	25	15	53*	1.2	230
1α(OH)D5	50	15	47*	0.8*	226

* Significantly different from control ($P < 0.05$).

3.2. Selectivity of 1α(OH)D5 action for transformed cells

We compared the growth effects of 1α(OH)D5 in various steroid receptor-positive as well as negative breast epithelial cell lines. These cell lines included (1) non-tumorigenic MCF12F breast epithelial cells, (2) ER$^+$, PgR$^+$, VDR$^+$, BT474, and MCF7 cells, and (3) ER$^-$, PgR$^-$, and VDR$^-$ highly metastatic MDA-MB-435 and MDA-MB-231 breast cancer cell lines. The results showed that both 1,25(OH)$_2$D$_3$ and 1α(OH)D5 were efficacious in suppressing cell proliferation of ER$^+$, PR$^+$, and VDR$^+$ BT474, T47D, ZR75, and MCF7 breast cancer cells. These compounds induced differentiation of ER$^-$, PgR$^-$, VDR$^+$, and BCA-4 cells [35] but did not show any growth effects in MDA-MB-435 and MDA-MB-231 cells. Other researchers have also reported similar results with other Vitamin D analogs [36]. Although our results indicate that the presence of VDR is necessary to potentiate Vitamin D's effect, it does not explain the lack of Vitamin D's effect on MCF12F cells that express low levels of VDR.

In order to examine whether 1α(OH)D5 selectively inhibits cell proliferation in transformed cells only, we evaluated the effects of 1α(OH)D5 on non-tumorigenic breast epithelial cells and compared them to the effects on BT474 breast cancer cells.
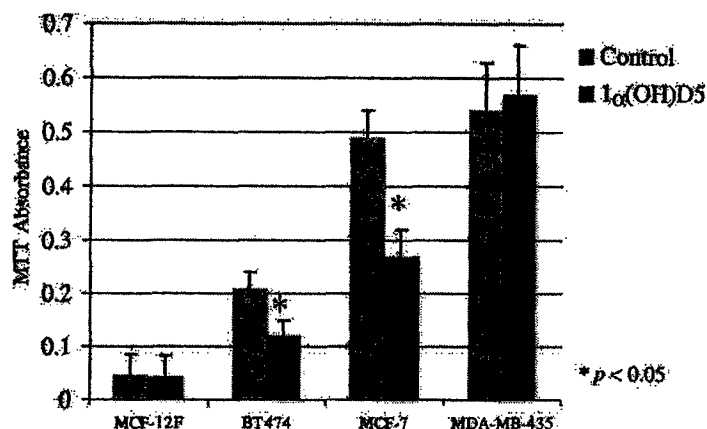
Fig. 6. Effects of $1\alpha(OH)D_5$ on viability of non-tumorigenic and cancer breast epithelial cells. Different cell lines were treated with $1\,\mu M$ $1\alpha(OH)D_5$ for 2 days and incubated with MTT for 2 h. The cells were lysed and washed prior to reading absorbance at 550 nm. MTT absorbance is proportional to the number of live cells. Each experiment was repeated twice and differences between the mean were assessed using student's t-test.

As shown in Fig. 6, incubation of MCF12F breast epithelial cells for 6 days with $1\alpha(OH)D_5$ at $1\,\mu M$ concentration did not result in suppression of cell proliferation as determined by the MTT absorbance assay. On the other hand, there was a significant inhibition of proliferation in both MCF7 and BT474 cells with $1\alpha(OH)D_5$ treatment. These results suggested that the effect of Vitamin D analog might be selective for transformed cells. The antiproliferative effects of $1\alpha(OH)D_5$ were also evident in in vivo experiments. Xenograft of ER^+, PgR^+, VDR^+, MCF7, ZR75/1, and BT474 cells or ER^-, PgR^-, VDR^+, and BCA-4 cells responded to $12.5\,\mu g$ $1\alpha(OH)D_5/kg$ diet and showed suppressed growth of these cells in athymic mice [35].

To confirm the selectivity of $1\alpha(OH)D_5$ for transformed breast cancer cells, we conducted three separate experiments. In the first experiment, we compared the efficacy of $1\alpha(OH)D_5$ between MCF12F cells with that of MNU-transformed MCF12F ($MCF12F_{MNU}$) cells. The $MCF12F_{MNU}$ cells have recently been established in our laboratory (unpublished data). The $MCF12F_{MNU}$ cells have altered morphology and growth properties as well as different growth factor requirements (Hussain and Mehta, unpublished data). Incubation of MCF12F and $MCF12F_{MNU}$ with $1\,\mu M$ $1\alpha(OH)D_5$ for 6 days resulted in 50% growth inhibition in $MCF12F_{MNU}$ cells without having any significant effects on MCF12F growth.

In a second study using the MMOC model, the effects of $1\alpha(OH)D_5$ were determined in mammary glands. Mammary glands respond to growth-promoting hormones and develop structurally differentiated alveoli within 6 days in culture. Incubation of glands with $1\,\mu M$ $1\alpha(OH)D_5$ for 6 days did not affect the growth-promoting effects of insulin, prolactin, aldosterone, hydrocortisone, estrogen, and progesterone (Fig. 7). Contrarily, $1\alpha(OH)D_5$ showed excellent anti-proliferative effects against DMBA-induced MAL and MDL (Fig. 5).

Experiments to determine the selectivity of $1\alpha(OH)D_5$ action against transformed cells were further extended to human tissues. The effects of $1\alpha(OH)D_5$ on the explants derived from normal breast tissues were compared with those of cancer tissue. Breast tissue samples were obtained from women undergoing mastectomy or lumpectomy at the University of Illinois at Chicago Hospital. Tissue explants of tumors and normal adjacent cells were incubated for 72 h in the MEME containing 5% fetal calf serum with or without $1\alpha(OH)D_5$ at $1\,\mu M$ concentration. Tissue sections were histopathologically evaluated, and Ki 67 expression was determined. Results showed that the histopathology of control and $1\alpha(OH)D_5$-treated normal breast tissue was identical with no difference in apoptosis or Ki 67 expression. On the other hand, the histological sections of the cancer tissue explants showed extensive apoptosis within the tissue with
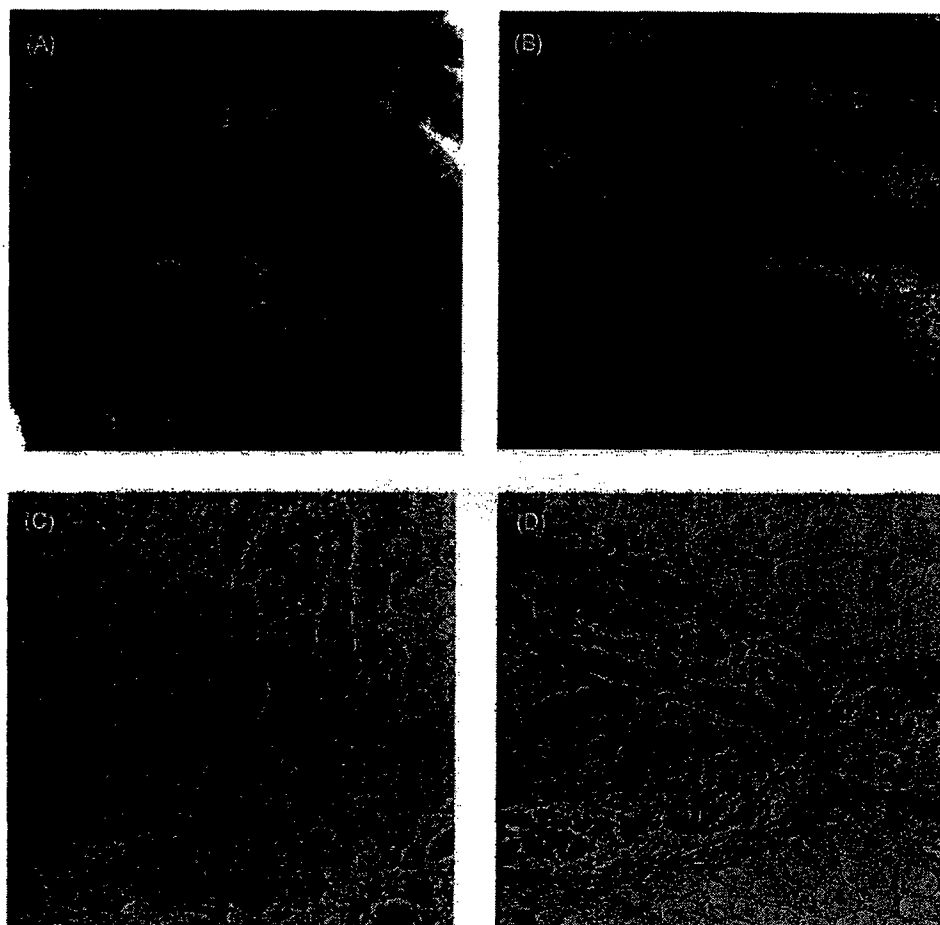
Fig. 7. The 6-day mouse mammary organ culture (MMOC) was performed without the carcinogen treatment. The data shows similar growth in both the control and $1\alpha(OH)D_5$ treated glands. (A) control; (B) $1\alpha(OH)D_5$; fixed and stained with carmine; (C) control and (D) $1\alpha(OH)D_5$, fixed, sectioned, and stained with H and E.

condensed chromatin and reduced Ki 67 expression after 72-h incubation with $1\alpha(OH)D_5$ (Mehta, unpublished data). Taken together, these results indicate that, in human breast epithelial tissues, $1\alpha(OH)D_5$ is selective for its effects on pre-cancerous or cancer cells but shows no effect on normal breast epithelial cell growth.

3.3. Mechanism of $1\alpha(OH)D_5$ action

The effects of $1\alpha(OH)D_5$ have also been evaluated in several breast cancer cell lines [37]. Although these studies do not focus directly on chemoprevention, they do provide excellent insight into the mechanism of action of $1\alpha(OH)D_5$ and its efficacy as an anti-proliferative agent. We had reported that, in ER^+, PgR^+, breast cancer cells, $1\alpha(OH)D_5$ inhibited cell growth by inducing apoptosis as well as differentiation, whereas in ER^- but VDR^+ cells, it induced cell differentiation without the induction of apoptosis [35]. Similar results have also been reported by numerous investigators using other analogs of Vitamin D [38]. The data from these studies consistently reported that breast cancer cells expressing VDR respond to Vitamin D analogs. These results suggested that the mode of action of $1\alpha(OH)D_5$ depended not only on expression of VDR but also on the expression of ER and ER-inducible genes such as PgR.

The effects of $1\alpha(OH)D_5$ on cell cycle were determined using breast cancer cells. The BT474 cells

Table 3

Effects of $1\alpha(OH)D_5$ on cell cycle phases in breast epithelial cell lines

Types	G1 (%)	S (%)	G2 (%)	G1/G2 (%)
BT474				
Control	60.7	30.5	8.8	6.9
$1,25(OH)_2D_3$	71.6*	22.1	6.3	11.4
$1\alpha(OH)D_5$	85.7*	10.3	4.0	21.4
MCF7				
Control	61.2	28.6	10.1	6.1
$1,25(OH)_2D_3$	71.9*	19.3	8.8	8.2
$1\alpha(OH)D_5$	70.0*	20.4	9.6	7.3
MDAMB435				
Control	22.8	31.3	45.9	0.5
$1,25(OH)_2D_3$	21.1	33.0	45.3	0.5
$1\alpha(OH)D_5$	21.1	23.6	55.3	0.4
MCF12F				
Control	72.4	16.2	11.4	6.4
$1,25(OH)_2D_3$	61.1*	20.2	19.0	3.2
$1\alpha(OH)D_5$	67.3*	16.2	16.5	4.1

* Significantly different from control ($P < 0.05$).

were treated with $1\,\mu M$ $1\alpha(OH)D_5$ for various time points and processed for FACS analysis. Results showed that 70% of the control cells were distributed in the G1 phase, whereas treatment with $1\alpha(OH)D_5$ induced growth arrest with 84% cells in the G1 phase of the cycle. The results are summarized in Table 3. In agreement with our cell proliferation data, there was no difference between the distribution of cells in various cell cycle stages for MCF12F and MBA-MD-231 cells with $1\alpha(OH)D_5$ treatment. Both MDA-MB-231 and MDA-MB-435 cells are devoid

of steroid receptors; therefore, these cells were not expected to respond to $1\alpha(OH)D_5$ treatment. These results further confirm that the action of $1\alpha(OH)D_5$ may be mediated, in part, by VDR.

The mechanism of action of $1\alpha(OH)D_5$ was further evaluated by determining the ability of the cells to undergo apoptosis. The BT474 cells were treated with $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 72 h and then stained with acridine orange and observed under fluorescent microscope for detection of chromatin condensation. Fig. 8 shows that BT474 cells underwent apoptosis with $1\alpha(OH)D_5$ treatment as determined by acridine orange and ethidium bromide staining. The stain distinguishes live cells from those that are undergoing apoptosis. On the other hand, no apoptosis was observed in ER^-, PgR^-, VDR^+, BCA-4 cells, though there was an induction of differentiation as shown by casein, lipids, and $\alpha2$ integrin expression [35].

Chemopreventive agents are being developed mostly for people who do not yet have disease but are at high risk of developing cancer. Here, we show that the Vitamin D analog might be selective for transformed cells. The population at high risk of developing cancer is assumed to be initiated for carcinogenesis and, as we have shown, initiated cells respond well to $1\alpha(OH)D_5$. In addition, we also showed here that $1\alpha(OH)D_5$ is effective against steroid-responsive cancer cells. These results suggest that $1\alpha(OH)D_5$ can be considered as a possible chemopreventive and therapeutic agent. Moreover, if given in combination with other agents, it may provide synergistic protection.

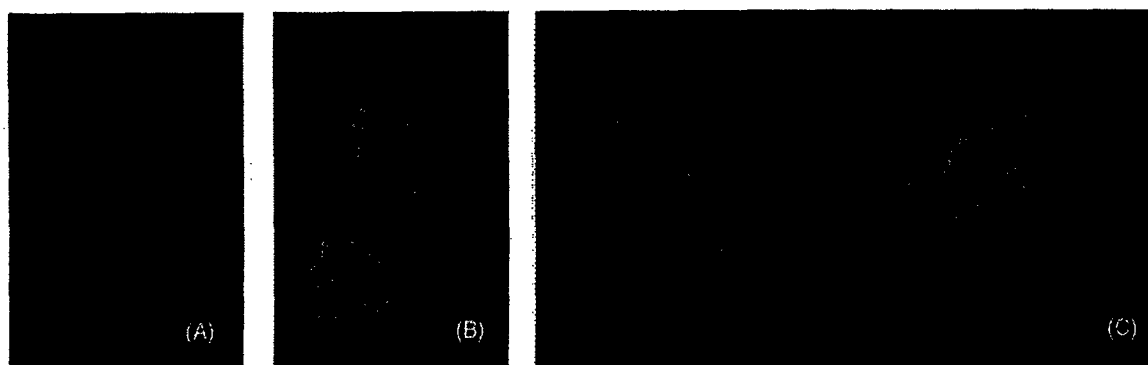It is unclear as to where chemoprevention ends and chemotherapy begins. However, the clear principle

Fig. 8. Induction of apoptosis in BT474 cells by $1\,\mu M$ $1\alpha(OH)D_5$, as determined by acridine orange and ethidium bromide staining. (A) control; (B) $1,25(OH)_2D_3$ (0.1 μM); (C) $1\alpha(OH)D_5$ (1 μM).

and prerequisite of chemoprevention is that the agent should not have any adverse effects. The lack of toxicity of $1\alpha(OH)D_5$ at an effective concentration may provide a rationale for its role in chemoprevention and therapy.

In summary, we have described here the chemopreventive properties of a relatively new non-toxic analog of Vitamin D, $1\alpha(OH)D_5$, against mammary carcinogenesis models. In addition, our results suggest that $1\alpha(OH)D_5$ may be active selectively against transformed cells without showing adverse effects on normal breast epithelial cells.

Acknowledgements

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Induction of differentiation by 1α-hydroxyvitamin D5 in T47D human breast cancer cells and its interaction with vitamin D receptors

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Abstract

The role of the active metabolite of vitamin D, 1,25 dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$), in cell differentiation is well established. However, its use as a differentiating agent in a clinical setting is precluded due to its hypercalcaemic activity. Recently, we synthesised a relatively non-calcaemic analogue of vitamin D_5, 1α-hydroxyvitamin D_5 (1α(OH)D$_5$), which inhibited the development of carcinogen-induced mammary lesions in culture and suppressed the incidence of chemically induced mammary carcinogmas in rats. In the present study, we determined the differentiating effects of 1α-(OH)D$_5$ in T47D human breast cancer cells and compared its effects with 1,25(OH)$_2$D$_3$. Cells incubated with either 10 or 100 nM of the analogues inhibited cell proliferation in a dose-dependent manner, as measured by the dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. Similar growth-inhibitory effects were also observed for MCF10$_{neo}$ cells. Both vitamin D analogues induced cell differentiation, as determined by induction of casein expression and lipid production. However, MCF10$_{neo}$ cells failed to respond to either vitamin D analogue and did not undergo cell differentiation. Since the cell differentiating effect of vitamin D is considered to be mediated via the vitamin D receptor (VDR), we examined the induction of VDR using reverse transcriptase–polymerase chain reaction (RT-PCR) in both cells. The results showed that, in T47D cells, both 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ induced VDR in a dose-dependent manner. Moreover, both analogues of vitamin D upregulated the expression of vitamin D response element-chloramphenicol acetyl transferase (VDRE-CAT). These results collectively indicate that 1α-(OH)D$_5$ may mediate its cell-differentiating action via VDR in a manner similar to that of 1,25(OH)$_2$D$_3$. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Vitamin D; Breast cancer cells; Differentiation; T47D; MCF10

1. Introduction

The research on vitamin D_3 and related compounds is currently at its apex. A vast amount of evidence has been collected, implicating the essential involvement of vitamin D metabolites in several cellular processes. The active metabolite 1,25 dihydroxy vitamin D_3 (1,25(OH)$_2$D$_3$) and related compounds suppress the development and progression of breast cancer and other carcinomas *in vivo* [1,2], inhibit the metastatic spread of tumour cells [3–5], and promote differentiation of breast cancer cells [6–8]. However, the calcaemic side-effects of 1,25(OH)$_2$D$_3$ have prevented its application as a phar-

maceutical agent. In recent years, considerable attention has been given to the development of vitamin D_3 analogues capable of inducing cell differentiation without systemic hypercalcaemia [8–10]. Many structural modifications are known to enhance several-fold the differentiating potency of vitamin D_3 analogues in normal (usually keratinocyte) or malignant (usually leukaemia) cell lines. Little attempt, however, has been made to evaluate vitamin D analogues of other series such as vitamin D_2, D_4, D_5 and D_6. This structural classification is based on the differences encountered in the side chain. Earlier studies reported that vitamin D_5 was the least toxic of vitamins D_2 through to D_6 [11].

During the past 2 years, we have been studying the role of 1α-hydroxyvitamin D_5 (1α(OH)D$_5$), an analogue of vitamin D_5 (24-ethyl-vitamin D_3), on breast cancer cell differentiation. We have characterised its calcaemic

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activity in vitamin D-deficient Sprague–Dawley rats [12]. The analogue $1\alpha(OH)D_5$ was synthesised from sitosterol acetate and was found to be less calcaemic than vitamin D_3. It was observed that $1\alpha(OH)D_5$ was effective against the development of carcinogen-induced mammary lesions in mouse mammary gland organ cultures [12]. In a more recent study, we observed that $1\alpha(OH)D_5$ inhibited incidence and tumour multiplicity of N-methyl-N-nitrosourea-induced mammary adenocarcinoma in rats (data not shown). These results clearly demonstrate that this vitamin D analogue might be a good candidate in the prevention of mammary carcinogenesis. In the present study, we evaluated the effects of $1\alpha(OH)D_5$ on cell differentiation and proliferation in oestrogen receptor (ER)-positive T47D breast cancer cells and compared the effects of the D_5 analogue with the active metabolite of vitamin D_3, $1,25(OH)_2D_3$. Moreover, we compared the effects of vitamin D analogues between ER+ T47D cells and ER- MCF10$_{neo}$ cells. Both cell lines are negative for functional p53 [13].

It is well known that the nuclear activity of vitamin D_3 is based on the interaction of the vitamin D active metabolite, $1,25(OH)_2D_3$, with the vitamin D receptor (VDR) [14]. The VDR is a nuclear receptor that belongs to the superfamily of ligand-dependent transcription factors and is expressed in all the vitamin D target tissues. VDR mediates its action by conjugating with the Retinoid X Receptor (RXR) [15–17]. The VDR-RXR dimer, once formed, is capable of recognising the vitamin D response element (VDRE) in the promoter region of the gene. The VDRE is composed of direct repeats of 6 DNA bases separated by 3-base intervening sequences [18]. Vitamin D appears to play an important role in stabilising and transactivating the VDR/RXR–VDRE complex [19,20]. Its interaction with VDR, therefore, represents the central step in the transmission of a signal to the transcription machinery, resulting in activation or suppression of transcription of genes leading ultimately to differentiation. We recently showed that the normal human breast epithelial cells lacking functional VDR do not respond to vitamin D to induce cell differentiation. However, transient transfection of VDR in these HBL-100 cells resulted in increased association of VDR-VDRE, as measured by the CAT reporter assay [21]. In the present study, we compared the effects of $1\alpha(OH)D_5$ and $1,25(OH)_2D_3$ on the transactivation of VDR–VDRE in T47D cells.

2. Materials and methods

2.1. Cells

The breast epithelial cell line, MCF10$_{neo}$, and human breast cancer cell line, T47D, were obtained from the American Type Culture Collection (Rockville, MD, USA). The MCF10$_{neo}$ cells were maintained in minimum essential medium with Earl's salts (MEME) medium supplemented with 10% fetal bovine serum (FBS), whereas T47D cells were maintained in RPMI supplemented with 0.2 I.U. bovine insulin/ml and 10% FBS. The monkey renal cancer CV-1 cells were maintained in MEME with 10% FBS supplement.

2.2. MTT assay

The cells were seeded in a 96-well/plate at a density of 500 cells/well in 100 μl/well of cell culture medium supplemented with 10% steroid-stripped serum. 24 h after seeding, the cells were incubated with 10 and 100 nM concentrations of $1,25(OH)_2D_3$ and $1\alpha(OH)D_5$, respectively. The medium was changed every 3 days. After 7 days, the cultures were used for the dimethylthiazolyl-2,5-diphenyltetrazolium Bromide (MTT) assay. MTT (5 mg/ml in phosphate buffered serum (PBS)) was added to the wells (15 μl/well) and incubated at 37°C for 2 h. The stop solution (20% sodium dodecyl sulphate (SDS) in 50% N,N-dimethylformamide) was then added (100 μl/well) and incubated for an additional 2 h. The plates were scanned at 590 nm OD, and the results for each treatment group were averaged.

2.3. Immunohistochemistry

MCF10$_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS and fixed in 10% formalin for 5 min. The fixed cells were further incubated in cold methanol for 3 min and acetone for 2 min. After blocking the cells with a protein block/normal goat serum (BioGenex, San Ramos, CA, USA), they were incubated with casein antibody (100 μg/ml) (Accurate Chemical and Scientific Corp. Westbury, NY, USA) for 2 h. The cells were then incubated with secondary anti-mouse biotinylated antibody for 30 min, followed by streptavidin–peroxidase complex and 3,3'-diaminobenzidine (DAB) solution as chromogen. Appropriate controls were performed to rule out non-specific staining with secondary antibody.

2.4. Lipid assay

MCF10$_{neo}$ and T47D cells were grown to subconfluence on coverslips for 7 days, washed in PBS, and fixed by incubating in cold methanol for 3 min and propylene glycol for 2 min. The cells were, at this point, stained with Oil Red O' for 30 min and rinsed in isopropyl alcohol then de-ionised water. Haematoxylene staining for 30 s and Scott solution rinse completed the assay.

2.5. RNA isolation and RT-PCR

The cells were incubated with 1, 10 or 100 nM $1,25(OH)_2D_3$ or $1\alpha(OH)D_5$ for 3 days. The medium

from the tissue culture flasks was removed and the cells were treated with RNA-Zol B (Tel-Test Inc., Friendswood, TX, USA). RNA were isolated according to the manufacturer's instruction and quantified spectrophotometrically. Reverse transcription (RT) and PCR were carried out using Advantage RT for PCR and Advantage cDNA PCR kit (Clontech Inc., Palo Alto, CA, USA). Primer sequences for VDR were selected and custom-synthesised by Oligos Etc. The sense primer was 5'-GGA GTT GCT GTT TGT TTG AC, and the antisense primer was 5'-CTT CTG TGA GGC TGT TTT TG. The primer for the housekeeping gene G3PDH was purchased from ClonTech. The touchdown PCR procedure was employed with minor modifications [22]. The first strand cDNA was heated at 94°C for 1 min followed by denaturation at 94°C for 45 sec, annealing at 68–66–64–62–60°C for 45 sec each time and extension at 72°C for 2 min, for 26 cycles. The final cycle was followed by a 7-min extension step at 72°C to ensure that the amplified DNA was double stranded. The absence of contaminant was routinely checked by RT-PCR assays of negative control samples (sterile buffer, provided in the kit). The PCR products were separated on 1.5% agarose gel at 64 volts for 3 h, stained with ethidium bromide and visualised by ultraviolet (UV)-transillumination.

2.6. Transient transfection

The reporter construct VDRE-tk-CAT was prepared by inserting a copy of VDRE into the BamHI site of the pBLCAT$_2$ as previously described [23]. For transfection, 1×10^5 CV-1 cells were plated in 24-well plates. Transfections were carried out using the calcium phosphate precipitation procedure. Briefly, 100 ng of VDRE-tk-CAT reporter plasmid, 250 ng of β-galactosidase (β-gal) expression vector, and 500 ng of VDR expression vectors were mixed with carrier DNA (pBluescript) to 1 µg of total DNA per well. The CAT activity was normalised for transfection efficiency by the corresponding β-gal activity.

3. Results

3.1. Effect of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on cell proliferation

The breast epithelial cells MCF10$_{neo}$ and breast cancer cells T47D were incubated with the vitamin D analogues for 7 days in culture. After this, the effects of vitamin D analogues were evaluated by the MTT assay. The results indicated a 31% and 50% growth inhibition for MCF10$_{neo}$ at 10 and 100 nM of 1,25(OH)$_2$D$_3$ concentrations, respectively, as compared with 50% and 72% inhibition with 1α(OH)D$_5$ at 10 and 100 nM,

respectively (Fig. 1a). The ER-positive, T47D cells showed a 29% and 52.5% growth inhibition after being exposed for 7 days to 1,25(OH)$_2$D$_3$ at 10 and 100 nM, respectively. Unlike MCF10$_{neo}$ cells, T47D cells did not exhibit increased growth suppression when exposed to 1α(OH)D$_5$. Both analogues suppressed growth of T47D cells by approximately 30% and 50% at low and high concentrations, respectively (Fig. 1b). These results suggest that both 1α(OH)D$_5$ and 1,25(OH)$_2$D$_3$ are comparable in producing antiproliferative effects in breast cancer cells.

3.2. Induction of differentiation of breast cancer cell lines

Since one of the major recognised functions of vitamin D is induction of cell differentiation, we evaluated the effects of both analogues on the induction of differentiation in both cell lines. As markers of cell differentiation, we used casein and lipid. Casein expression was measured by immunocytochemistry using casein antibodies. Results showed that, for T47D cells, casein was expressed in less than 10% of the control cells.
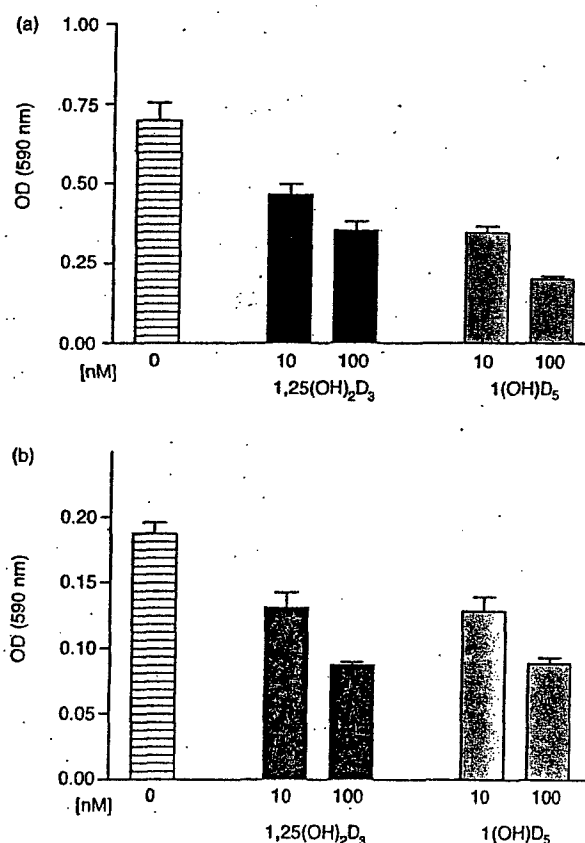

Fig. 1. Effects of 1,25(OH)$_2$D$_3$ and 1α (OH)D$_5$ on the proliferation of MCF10$_{neo}$ cells and T47D cells. The MTT assay was carried out using duplicate cultures and the experiments were repeated three times. The error bars represent the standard deviation. (a) MCF10$_{neo}$ cells; (b) T47D cells.

CONTROL   1,25(OH)$_2$D$_3$ [10nM]   1(OH)D$_5$ [10nM]
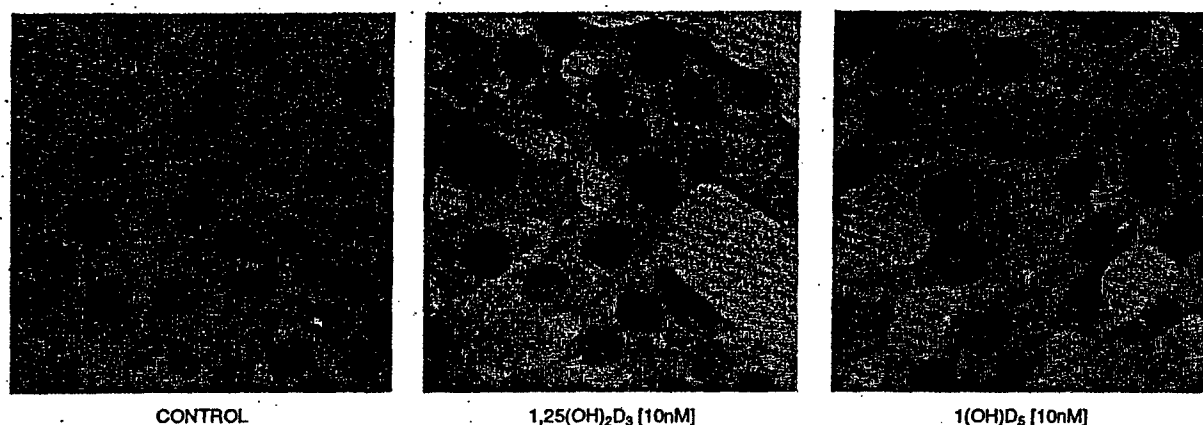
Fig. 2. Effect of vitamin D analogues on casein expression in T47D cells. Immunohistochemical staining for casein expression was carried out as previously described in the presence or absence of the vitamin D analogues.

After 7 days treatment with 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$, the intensity and number of cells expressing casein increased to approximately 70 and 85% at 10 and 100 nM concentrations, respectively. No difference was noticed between the effects of D$_3$ or D$_5$ analogues (Fig. 2 and data not shown). Similarly, there was a dramatic increase in the expression of lipid production in T47D cells after 7 days of treatment with 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ (Fig. 3). These results indicated that both vitamin D analogues induce cell differentiation in T47D cells. In contrast, the MCF10$_{neo}$ cells, tested for the same markers of differentiation, did not show any presence or induction of either casein or lipids in the control cells or in cells exposed to vitamin D$_3$ or D$_5$ (data not shown).

3.3. Transactivation of VDRE

The VDRE transactivation activity of the vitamin D analogues was determined using the CAT reporter gene containing VDRE (VDRE-tk-CAT). In order to compare the activity of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ for transactivating the VDRE reporter gene, we selected monkey renal cancer cells (CV-I). These cells lack a functional VDR, so one can evaluate the binding activity of vitamin D analogues only in the transiently transfected VDR. The active metabolite of vitamin D, 1,25(OH)$_2$D$_3$, should not show any increase in CAT activity if the cells are transfected only with VDRE-tk-CAT. As shown in Fig. 4, neither vitamin D$_3$ nor vitamin D$_5$ analogues could induce CAT activity, indicating a lack of endogenous VDR in these cells. However, when 500 ng VDR (Fig. 4b) was co-transfected with VDRE and the cells were incubated with 10 or 100 nM of 1,25(OH)$_2$D$_3$ or 1α(OH)D$_5$, there was enhanced expression of the CAT reporter gene. These results clearly indicate that both analogues of vitamin D can bind to the VDR and the complex can bind to the VDRE to initiate signal transduction. However, the extent of VDRE-reporter transactivation was 7- to 8-fold greater when the transfected cells were incubated with 1,25(OH)$_2$D$_3$ at 10 nM and nearly 2-fold greater at 100 nM, respectively, compared with 1α(OH)D$_5$ at the same concentrations. This is consistent with the observed
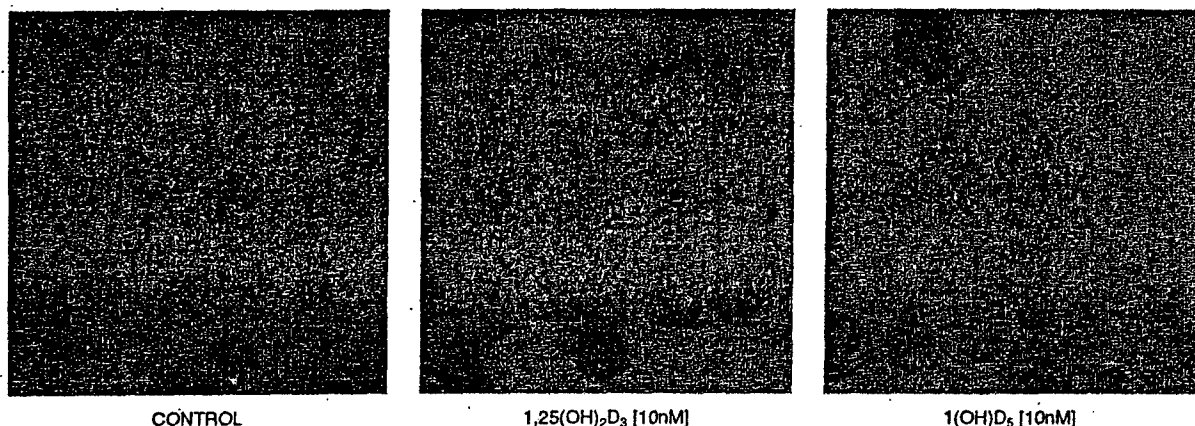

CONTROL   1,25(OH)$_2$D$_3$ [10nM]   1(OH)D$_5$ [10nM]

Fig. 3. Effects of 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ on lipid expression in T47D cells. A lipid assay was carried out as previously described in the presence or absence of vitamin D analogues.

finding that a log molar higher concentration of 1(OH)D$_5$ is needed to obtain an equivalent response to that observed with 1,25(OH)$_2$D$_3$.

3.4. Induction of VDR mRNA as determined by RT-PCR

Experiments were carried out to determine if VDR mRNA is induced by the vitamin D analogues in T47D and MCF10$_{neo}$ cells. Total RNA from the cells was isolated and reverse-transcribed. The cDNA was amplified using Taq polymerase and separated on 1.5% agarose gel. As shown in Fig. 5, the housekeeping gene G3PDH (C) was identical for all the cDNAs, indicating an equal loading of the gels. The VDR separated as a 420 bp fragment on the gel. As shown in Fig. 5(a), in T47D cells, there was a basal level of expression of VDR; however, incubation of cells for 3 days with either 10 or 100 nM of 1,25(OH)$_2$D$_3$ increased the VDR expression in a dose-related manner. Similar results

were also obtained with 1α(OH)D$_5$, as shown in Fig. 5(a). In contrast, MCF10$_{neo}$ cells expressed the basal level of VDR in the cells; but, there was no induction of VDR message by the vitamin D analogues (Fig. 5b). These results indicate that the lack of induction of differentiation by vitamin D in MCF10$_{neo}$ cells may be related to a lack of induction of VDR in these cells by vitamin D analogues.

4. Discussion

The effects of vitamin D analogues as differentiating agents and inhibitors of cell proliferation for breast cancer cells have been reported [1,7]. It is generally believed that the cells expressing VDR often respond to vitamin D analogues, whereas cells such as MDA-MB-231, which are ER- and express low or non-detectable
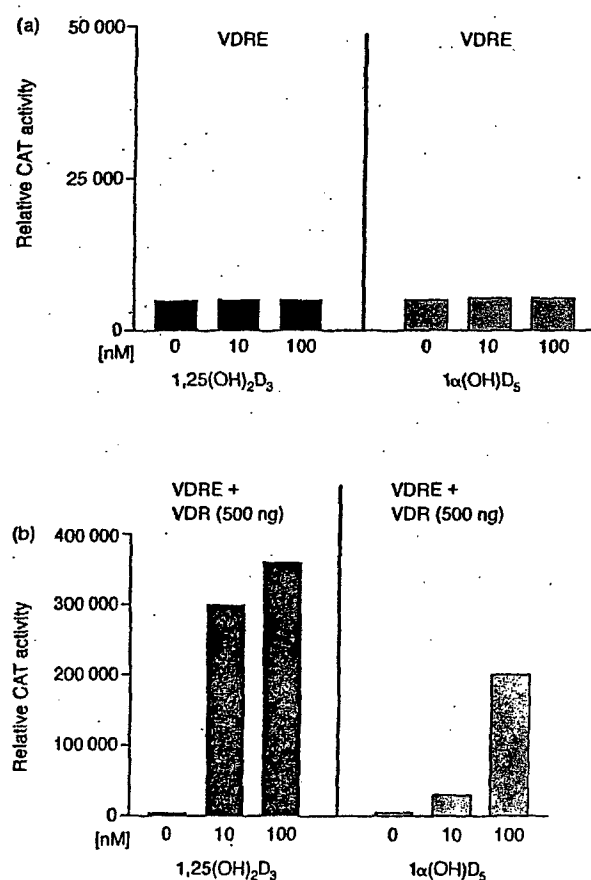

Fig. 4. Effects of 1,25(OH)D$_3$ and 1α(OH)D$_5$ on the transactivation of the VDRE-conjugated reporter gene. Transient transfections of CV-1 cells with either VDRE-tk-CAT alone (a) or with VDR (b) was carried out by the calcium phosphate precipitation procedure. The cells were incubated with 10 and 100 nM vitamin D analogues for 3 days. CAT activity was measured spectrophotometrically. The experiments were repeated twice.
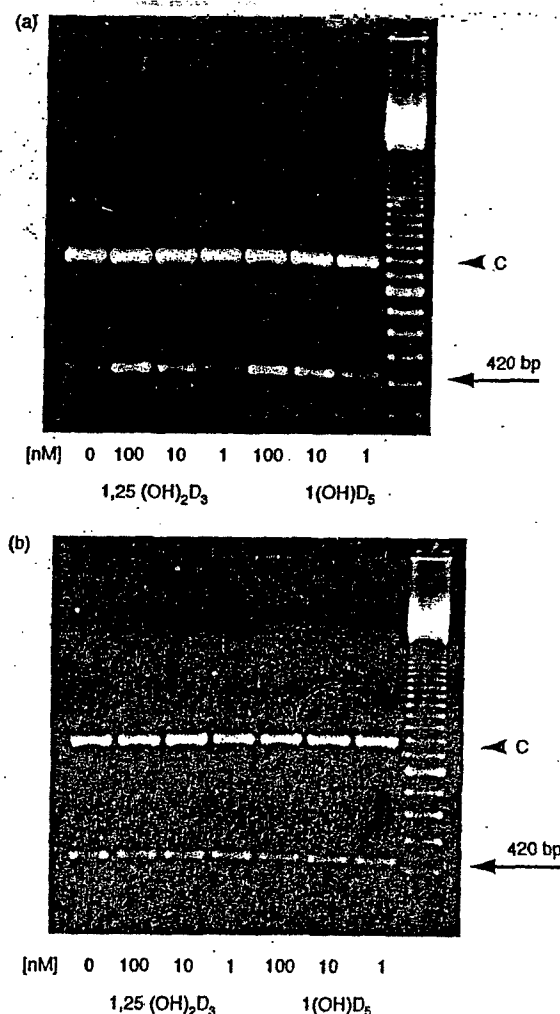
Fig. 5. Effects of vitamin D analogues on the expression of VDR mRNA in MCF10$_{neo}$ and T47D cells. Cells were incubated with various concentrations of analogues for 3 days in culture as previously described. VDR expression was measured by RT-PCR in (a) T47D cells; (b) MCF10$_{neo}$ cells. C, control housekeeping gene.

levels of VDR, do not respond to active vitamin D analogue(s) [24]. The VDR-mediated transcription regulatory genes include *TGFβ*, *EGF*, *c-myc* [25,26], and cell cycle regulators. The effects of various vitamin D analogues on programmed cell death have been evaluated in a variety of breast cancer cell lines. Consistently, MCF-7 cells which are ER+, VDR+ and positive for wild-type p53 exhibit apoptosis in response to vitamin D [27,28]. Although considerable literature exists for vitamin D-induced differentiation, its clinical application has been limited. This is due to its cytotoxicity at the concentration that induces differentiation. To this end, we have identified an analogue of the vitamin D_5 series which is non-calcaemic at the concentration at which 1,25-dihydroxyvitamin D_3 would induce hypercalcaemia. We previously reported that 1α-hydroxyvitamin D_5 inhibits carcinogen-induced development of mammary lesions in culture [12]. We also reported that it induces VDR and TGFβ in mammary epithelial cells. In this report, we addressed the question, "Does 1-hydroxyvitamin D_5 induce cell differentiation of breast cancer cells to the same extent as the active metabolite of vitamin D, 1,25-dihydroxyvitamin D_3?". T47D and MCF10$_{neo}$ cells were selected for the present study, since T47D cells are ER- and progesterone (PR)-positive and MCF10$_{neo}$ cells are negative for both ER and PR. Both analogues of vitamin D, 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$, inhibited cell proliferation to the same extent and induced differentiation as determined by the increased expression of differentiation markers.

The MCF10$_{neo}$ cells were originally derived from normal breast tissue and the epithelial cells were subsequently immortalised. The MCF10$_{neo}$ cells are ER-VDR+ and stably transfected with ras. The cells are tumorigenic in athymic mice. Since both T47D and MCF10$_{neo}$ have similar VDR and p53 status and differ only in their ER status, we compared the response of T47D ER+ and MCF10$_{neo}$ cells to two analogues of vitamin D. The MCF10$_{neo}$ cells, like T47D cells, exhibited a suppression of cell proliferation; however, no induction of differentiation was noticed. This, therefore, raised the question of whether induction of VDR is essential for cell differentiation. We evaluated the induction of VDR mRNA by these two vitamin D analogues. The results showed that MCF10$_{neo}$ cells constitutively expressed VDR-mRNA. However, there was no induction of the VDR message by either of the vitamin D analogues. In contrast, there was a dose-dependent increase in the expression of VDR mRNA in the T47D cells by both vitamin D_3 and D_5 analogues. These results suggest that there may be a positive association between the differentiation of cells by vitamin D and the induction of vitamin D-induced mRNA of VDR. Alternatively, the antiproliferative effects may be mediated by p53 although this is most unlikely in this case as both MCF10$_{neo}$ and T47D cells do not have functional

p53 [10] and yet they respond to antiproliferative activity of vitamin D analogues. These results suggest that the antiproliferative effects and differentiating effects of vitamin D analogues may be independent of the cellular p53 status. These results are consistent with a recent report indicating the non-involvement of p53 in vitamin D-mediated differentiating/cell growth suppressing functions in breast cancer cells. Thus, it is not clear what mechanism may be operative for the suppression of cell growth by vitamin D analogues. If both antiproliferative effects and cell differentiating effects are mediated by VDR, then it is possible that the constitutive level of VDR will be sufficient to mediate vitamin D's effects in suppressing cell proliferation but that induction of new VDR mRNA may be necessary for cell differentiation.

Comparison between the action of a natural ligand of vitamin D, 1,25(OH$_2$)D$_3$, and a vitamin D_5 analogue was also made in terms of their ability to transactivate a VDRE-reporter *CAT* gene. We selected VDR-negative CV-1 cells for these studies so that the endogenous VDR would not interfere with the interpretation of data. Since CV-1 cells are truly VDR-negative, they do not respond to incubation with 1,25(OH$_2$)D$_3$ and do not transactivate the VDRE-CAT reporter. Since both T47D and MCF10$_{neo}$ cells express basal levels, to different extents, of VDR, the vitamin D analogue-induced transactivation of the CAT reporter may vary between these two cells and will compromise comparing the two analogues of vitamin D. Results showed that both 1,25(OH)$_2$D$_3$ and 1α(OH)D$_5$ bind VDR and interact with VDRE. It was noted that, with 500 ng VDR transfection into CV-1 cells, 1,25(OH)D$_3$ at 10 nM induced the reporter expression by more than 150-fold compared with the induction by 1α(OH)D$_5$ at the same concentration. The results indicate that, at equimolar concentrations, 1,25(OH)$_2$D$_3$ is more potent in transactivating the *VDRE* reporter gene than 1(OH)D$_5$. This is consistent with the earlier findings that, in mouse mammary gland organ cultures, the D_5 analogue is required at a log molar higher concentration to achieve similar effects to those observed with 1,25(OH)$_2$D$_3$. The advantage, however, is that the D_5 analogue does not induce unwarranted toxicity which is often associated with 1,25-dihydroxyvitamin D_3. These studies collectively indicate that the vitamin D_5 series of agents mediate their action via the same VDR-mediated mechanism that is operative with the active metabolite of vitamin D_3.

Acknowledgements

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